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4. Title of the invention — SUBSTANCES AND THEIR THERAPEUTIC USE

5. Name of your agent *(if you have one)* — MEWBURN ELLIS

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Substances and Their Therapeutic Use

Field of the Invention

The present invention relates to substances and their therapeutic use, and in particular to the identification of regions of $p21^{WAF1}$ that binds to cyclin dependent kinases and/or cyclin D1, and to substances, fragments and mimetics based on this region. The present invention also relates to pharmaceutical compositions comprising these molecules and their use in therapeutic applications for treating hyperproliferative disorders, such as cancer and psoriasis, and compositions comprising these molecules and their use in applications relating to growth in eukaryotic cells.

Background of the Invention

The tumour suppressor function of p53 is linked to a DNA-damage inducible cell cycle checkpoint pathway (Kastan et al., 1991), in which p53 can induce either growth arrest (Agarwal et al., 1995) or apoptosis (Clarke et al., 1993; Lowe et al., 1993; Merritt et al., 1994) in the damaged cells. The biochemical activity of p53 most tightly associated with tumour suppression and growth arrest involves an ionising radiation-dependent activation of sequence-specific transcriptional activity (Kastan et al., 1991; Lu and Lane, 1993; Pietenpol, et al., 1994). p53 induces the transcription of a number of genes, the products of which play a direct role in mediating growth arrest. These p53-inducible negative regulators of cell proliferation include: the cyclin dependent kinase inhibitor (CKI), $p21^{WAF1}$ (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Gu et al., 1993); an apoptosis promoting protein, Bax (Miyashita and Reed, 1995); the insulin growth factor binding protein IGF-BP3 (Buckbinder et al., 1995); and Gadd45 (Kastan et al., 1992), a potent inhibitor of cell proliferation with an as yet unclear biochemical function (Kearsey et al., 1995).

A common event in the development of human neoplasia is the inactivation of a DNA damage-inducible cell cycle checkpoint pathway regulated by p53 (Hollstein et al.,1991; Lane, 1992; Agrawal et al., 1995). A variety of mechanisms can lead to the functional inactivation of the p53 pathway, including: missense mutations within, or deletions of the p53 gene, inactivation of wild type p53 protein function by interaction with the oncogenic cellular protein mdm-2 (Momand et al., 1992), or the inability to induce downstream effector molecules, such as $p21^{WAF1}$ (Deng et al., 1995; Waldman et al., 1995).

Our growing knowledge of the molecular mechanisms underlying the transformation of mammalian cells offers the opportunity to create rationally designed inhibitors of specific biochemical processes essential to uncontrolled cell proliferation or cancer. Recent developments have shown that the reactivation of the p53 pathway in some human tumours could in theory be achieved by: (i) activating the biochemical function of mutant p53 protein (Halazonetis and Kandil, 1993; Hupp et al., 1993), possibly using small peptides as leads for drug design (Hupp et al., 1995); (ii) disrupting the interaction of the oncogene mdm-2 and wild type p53 through the use of peptide-mimetic inhibitors of complex formation (Picksley et al., 1994); (iii) restoring or mimicking the function of the downstream effector molecule $p21^{WAF1}$, which on its own is capable of mediating growth suppression (El-Deiry et al., 1993; Eastham et al., 1995).

$p21^{WAF1}$ is an inhibitor of both the G1 cyclin dependent protein kinases (CDKs; which control the progression from G1 into S phase) (Harper et al., 1995) and proliferating cell nuclear antigen (PCNA; an essential DNA-replication factor) (Florez-Rozas et al., 1994; Waga et al., 1994). Thus, inhibition of the function of either CDKs or PCNA provides, in theory, two distinct avenues for development

of drug discovery programmes which are based on the activity of $p21^{WAF1}$. The PCNA binding function of $p21^{WAF1}$ can be mimicked by a 20-amino acid peptide derived from the C-terminal domain of $p21^{WAF1}$ and this peptide is sufficient to partially inhibit SV40 replication in vitro (Warbrick et al., 1995).

Despite its PCNA binding role, the primary function of the $p21^{WAF1}$ protein as a growth suppressor appears to be inhibition of the G1 cyclin-CDK complexes (Chen et al., 1995; Harper et al., 1995; Luo et al., 1995; Nakanishi et al., 1995b). The smallest portion of $p21^{WAF1}$ reported to act as a CDK-inhibitor in vitro is the N-terminal domain, composed of residues 1-75 (Luo et al., 1995), which inhibits cyclin E-Cdk2.

Summary of the Invention

The present invention concerns (i) the elucidation of the molecular mechanism of cyclin D1-Cdk4 complex inhibition by $p21^{WAF1}$, and (ii) the identification of peptide mimetics of $p21^{WAF1}$ inhibitory activity, through the examination of the binding and inhibitory properties of a series of synthetic peptides based on the amino acid sequence of $p21^{WAF1}$. Our studies found that two peptides derived from the N-terminal domain of $p21^{WAF1}$ have biochemical activity; a peptide 4 (residues 46-65) forms a stable complex with Cdk4, but has no inhibitory activity, while a peptide 2 (residues 16-35) binds to cyclin D1 and inhibits Cdk4 activity with a Ki of 2 μM.

These data define for the first time a cyclin binding site on $p21^{WAF1}$ and suggest that one mechanism involved in the CDK inhibitory action of $p21^{WAF1}$ employs binding to the cyclin subunit of the CDK holoenzyme. This has lead us to propose that $p21^{WAF1}$ can inhibit Cdk4 activity allosterically through conformational changes in the structure of cyclin D1.

Furthermore, peptides based on the C-terminal sequence of $p21^{WAF1}$ interact with both cyclin D1 and Cdk4, and are potent inhibitors of Cdk4 activity, with a peptide (peptide 10) composed of residues 141-160 having a Ki of 0.1 μM. We show that both of the inhibitory peptides bind at physiologically relevant sites on cyclin D1 and/or Cdk4, and that they display specificity mimicking that of full length $p21^{WAF1}$. Importantly, the potency of the C-terminal peptide is improved by making a single amino acid substitution (D - A at position 149). We have mapped the inhibitory component of this peptide using alanine mutation analysis and show that it is distinct from the PCNA interaction domain, which also resides in the C-terminal region of the $p21^{WAF1}$ protein.

Remarkably, a stretch of just five amino acids contains the Cdk4 inhibitory motif and a single conservative mutation at either of two hydrophobic amino acid residues completely abolishes the inhibitory activity of the peptide. These data have exciting implications for the mechanism of action of $p21^{WAF1}$ protein and represent a starting point for a drug design programme aimed at producing synthetic molecules functioning as tumour suppressors downstream of p53.

Accordingly, in one aspect, the present invention provides a substance which has the property of inhibiting cdk4, said substance comprising:

(i) a peptide fragment consisting of the motif xyLzF, wherein y and z are any amino acid and x is preferably R, or a derivative of said peptide fragment; or,

(ii) a functional mimetic of said peptide fragment.

In a further aspect, the present invention provides the above substance for use in a method of medical treatment.

In a further aspect, the present invention provides the use of a substance which has the property of inhibiting cdk4 in

the preparation of a medicament for the treatment of a hyperproliferative disorder, said substance comprising:

(i) fragment of the C-terminal portion of $p21^{WAF1}$, or an active portion or derivative thereof; or,

(ii) a peptide fragment including the motif xyLzF, wherein y and z are any amino acid and x is preferably R, or a derivative of said peptide fragment; or,

(iii) a functional mimetic of (i) or (ii).

In a preferred embodiment, the C-terminal portion of $p21^{WAF1}$ consisting of the peptide motif KRRLIFSK was found to completely inhibit cyclin-cdk4 activity and to prevent phosphorylation of pRb (see figure 8).

In a further aspect, the present invention provides a substance which has the property of binding to cdk4 for use in a method of medical treatment, said substance comprising:

(i) a fragment of the $p21^{WAF1}$ protein consisting of residues 46-65 of the $p21^{WAF1}$ amino acid sequence, or an active portion or derivative thereof; or,

(ii) a functional mimetic of said fragment.

In a further aspect, the present invention provides the use of a substance which has the property of binding cdk4 in the preparation of a medicament for the treatment of a hyperproliferative disorder, said substance comprising:

(i) a fragment of the $p21^{WAF1}$ protein consisting of residues 46-65 of the $p21^{WAF1}$ amino acid sequence, or an active portion or derivative thereof; or,

(ii) a functional mimetic of said fragment.

In a further aspect, the present invention provides a substance which has the properties of binding cyclin D and/or inhibiting cdk4 for use in a method of medical treatment, said substance comprising:

(i) a fragment of the $p21^{WAF1}$ protein consisting of residues 16-35 of the $p21^{WAF1}$ amino acid sequence, or an active portion or derivative thereof; or,

(ii) a functional mimetic of said fragment.

In a further aspect, the present invention provides the use of a substance which has the property of binding cyclin D1 and/or inhibiting cdk4 in the preparation of a medicament for the treatment of a hyperproliferative disorder, said substance comprising:

(i) a fragment of the $p21^{WAF1}$ protein consisting of residues 16-35 of the $p21^{WAF1}$ amino acid sequence, or an active portion or derivative thereof; or,

(ii) a functional mimetic of said peptide fragment.

In the present invention, "an active portion" means a peptide which is less than the fragment of the $p21^{WAF1}$ amino acid sequence, but which retains the relevant property mentioned above.

In the present invention, "functional mimetic" means a substance which may not contain an active portion of the $p21^{WAF1}$ amino acid sequence and is probably not a peptide at all, but which has the relevant property mentioned above.

In the present invention, "a derivative" means a peptide modified by varying its amino acid sequence, eg by manipulation of the nucleic acid encoding the peptide or by altering the peptide itself. Such derivatives of the natural amino acid sequence may involve insertion, addition, deletion or substitution of one or more amino acids, without fundamentally altering the essential activity of the peptides. An example of a derivative is the p21WAF1 mutant in which A was substituted for D at position 149 of the full length protein, this mutant having enhanced cyclin D1-cdk4 inhibitory activity.

In a further aspect, the present invention provides pharmaceutical compositions comprising one or more of the above substances in combination with a pharmaceutically acceptable carrier.

In a further aspect, the present invention relates to compositions comprising one or more of the above substances and their use in controlling the growth of eukaryotic cells, eg as a food preservative or as an agent to promote the growth of plants.

In a further aspect, the present invention provides compounds comprising any of the above substances coupled to carrier molecules, enabling the compounds to be delivered to cells *in vivo*. In one embodiment, the carrier molecule is a 16 aa peptide sequence derived from the homeodomain of *Antennapedia* (e.g. as sold under the name "Penetratin") which can be coupled to one of the above substances via a terminal Cys residue. Alternatively, as in the examples described below, a carrier peptide (having the sequence RQIKIWFQNRRMKWKK) can be synthesised so it is directly attached to peptide fragments. The "Penetratin" molecule and its properties are described in WO 91/18981.

In a further aspect, the present invention provides the use of the above substances in methods of designing or screening for mimetics of the substances.

Accordingly, the present invention provides a method of designing mimetics of $p21^{WAF1}$ having the biological activity of cdk4 binding or inhibition, the activity of allosteric inhibition of cdk4 and/or the activity of cyclin D1 binding, said method comprising:

(i) analysing a substance having the biological activity to determine the amino acid residues essential and important for the activity to define a pharmacophore; and,

(ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, eg by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, eg stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand

and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Brief Description of the Drawings

Figure 1. The Ability of Peptides from $p21^{WAF1}$ to Interact with Cdk4 and Cyclin D1.
Top panel: a list of the peptides 1-11 based on the sequence of $p21^{WAF1}$. Bottom Panel: The $p21^{WAF1}$ peptides were bound to streptavidin-agarose beads and added to reticulocyte lysates containing either Cdk4 or cyclin D1 labelled with [^{35}S]methionine. After extensive washing bound proteins were analysed using SDS-PAGE followed by autoradiography. The bands were quantified using a Bio-Imager and Whole Band Analysis Software (Millipore). The results are representative of 3 such experiments.

Figure 2. Addition of $p21^{WAF1}$ Based Peptides to Cyclin D1-Cdk4 Phosphorylation Assays.
Cyclin D1-Cdk4 assays were carried out in vitro using lysates from Sf9 insect cell following co-infection with Cdk4 and cyclin D1 baculovirus constructs and GST-Rb as the substrate. $p21^{WAF1}$ peptides (Figure 1; Top panel) were added

to the assays at a concentration of 17 μM and the effect on Cdk4 activity was assessed by SDS-PAGE and autoradiography. Top panel: autoradiograph; bottom panel: quantification of the autoradiograph using bio-imaging, relative binding is expressed in terms of Cdk4 activity in the absence of peptide. The data are representative of 4 experiments.

Figure 3. Quantification of Peptide Inhibition.
Peptides 4, 8, 2 and 10 were added to cyclin D1-Cdk4 assays at various concentrations between 0.01-34 μM. Top panel: a plot of activity (%) relative to Cdk4 activity measured in the absence of peptide against peptide concentration. Bottom panel: gives the Ki for each peptide. The data represent the mean of 3 experiments.

Figure 4. Interaction of Peptide with Cdk4 and Cyclin D1 in the Presence of $p21^{WAF1}$.
Top panel: The ability of $p21^{WAF1}$ to interfere with peptide 2 (A) and peptide 10 (B & C) binding to Cdk4 and/or cyclin D1 was determined by carrying out the peptide precipitation assay from reticulocyte lysates in the presence of 0, 0.5, 2 μg of $p21^{WAF1}$. Bottom panel: to determine if the inhibition of Cdk4 by $p21^{WAF1}$ could be relieved by the addition of a peptide 10 mutant, the R to A mutant (residue 15 of peptide 10) that was no longer an efficient inhibitor but still displayed partial binding activity, increasing concentrations of peptide (1, 5, 17 & 34 μM) were added to cyclin D1-Cdk4 GST-Rb phosphorylation assay in the presence of a fixed concentration of $p21^{WAF1}$ (50 μM). Panel A, shows the R to A peptide 10 mutant, and panel B uses peptide 6 (Figure 1) as a control.

Figure 5. Peptides 2 or 10 are Not Substrates for Cyclin D1-Cdk4.
Top panel: phosphorylation assays using peptides 2, 4 & 10. Bottom panel: phosphorylation of $p21^{WAF1}$ and GST-Rb were carried out as described in Experimental Procedures. Lane

1, 0.5 μg $p21^{WAF1}$ plus PKC; lane 2, cyclin D1- Cdk4 plus GST-Rb; lane 3, cyclin D1-Cdk4 plus GST-Rb and 0.5 μg p21WAF1; lane 4 cyclin D1-Cdk4 plus 0.5 μg $p21^{WAF1}$.

Figure 6. Cyclin B-Cdc2 Assays.
To determine if peptides 2 and 10 could inhibit cyclin B-Cdc2 kinase activity assays were performed using Sf9 cell lysates which were co-expressing human cyclin B and Cdc2. The conditions were identical to those described in the Experimental Procedures for cyclin D1-Cdk4 except that histone H1 (0.5 μg/assay) was used as the substrate. Cyclin D1-Cdk4 (panel A) and cyclin B-Cdc2 (panel B) were assayed in the presence of increasing concentrations of peptide 2 (0.25, 3, 10 and 40 μM) and peptide 10 (0.1, 0.5, 5, 20 μM).

Figure 7. Size Scan of Peptide 10.
Top panel: shows the sequences of a series of peptides based on peptide 10 designed to find the minimal inhibitory domain. The boxed residues represent the minimal inhibitory domain. Bottom panel: the peptides ware added to cyclin D1-Cdk4 assays and analysed by SDS-PAGE and autoradiography.

Figure 8. Alanine Scan Mutations of Peptide 10.
In order to pin point residues that were critical for the inhibition of Cdk4 by peptide 10 a series of point mutations were constructed in which each residue was sequentially changed to alanine. The peptides were added to cyclin D1-Cdk4 assays and the results were analysed by SDS-PAGE and autoradiography (**A**) then quantified using a Bio-Imager (**B**). The results are expressed relative to Cdk4 activity in the absence of peptide and are representative of 3 experiments. Having identified the critical residues we then synthesised an untagged eight amino acid peptide which contained the R, L and F (KRRLIFS). panel C shows the phosphorylation of GST-Rb by cyclin D1-Cdk4 in the

presence of increasing concentrations of this truncated peptide.

Figure 9. Comparison of Inhibitory Peptides with Full Length $p21^{WAF1}$ Protein.

Top panel: concentration curves for peptide 10, D to A mutant peptide 10, a p16INK4 derived peptide (Fahraeus et al., 1996) and full length his-$p21^{WAF1}$ were determined using the cyclin D1-Cdk4 assay analysed by SDS-PAGE, autoradiography and bio-imaging. Bottom panel: data for the Ki of each inhibitor. The results are the mean of 3 such experiments.

Figure 10. (A) Binding and Inhibitory Domains of $p21^{WAF1}$.

The hatched residues show the regions of $p21^{WAF1}$ identified in this study as being important for cyclin D1 and Cdk4 binding, and Cdk4 inhibition in the N-terminal domain, as well as a novel inhibitory domain in the C-terminus of $p21^{WAF1}$. The residues found to be important for the interaction of $p21^{WAF1}$ with PCNA (Warbrick et al., 1995) are shown in black. In addition, the smallest portion of $p21^{WAF1}$ that was found to inhibit CDK activity in vitro (Luo et al., 1995) prior to the present study is indicated.

(B) Model of the Allosteric Inhibition of Cyclin D1-Cdk4 Activity by $p21^{WAF1}$.

Pathway I proposes that $p21^{WAF1}$ binds first to Cdk4 through residues (46-65) maintaining a catalytically active complex. This stable and active complex could be related to the proposed role of $p21^{WAF1}$ as an assembly factor which promotes the formation of active cyclin-CDK complexes (Zhang et al., 1994). The induction of $p21^{WAF1}$ protein level in response to growth inhibitory signals would then lead to the binding of a second molecule of $p21^{WAF1}$, through residues 16-35, to the cyclin D1 subunit causing a change in the conformation of the cyclin which allosterically inhibits Cdk4 activity. In this model, it is not known

whether the binding of the second $p21^{WAF1}$ molecule is facilitated by the presence of the original molecule nor is it known whether the mechanism whereby cyclin D1 conformational changes are transmitted to the kinase involves rearrangement of the T-loop (Pines, 1995). Alternatively (pathway II), small peptide mimetics of $p21^{WAF1}$ (peptide 2) could bypass the physiological requirement for two molecules of $p21^{WAF1}$ to bind and inhibit cyclin D1-Cdk4 complex activity. This provides a basis for the design of small molecular weight compounds which would prevent aberrant cell proliferation by inhibiting key G1 cyclin-CDKs.

Figure 11. Introduction of $p21^{WAF1}$ based peptides into cells A series of synthetic peptides based on the sequence of peptide 10 (Peptides I, II and III above) were synthesised with carrier peptide (shaded sequences). The underlined residue in peptide-I is the M to A mutation which prevents PCNA binding. The peptides were added to proliferating HaCaT cells, grown in DMEM plus 10% FCS. The cells were incubated for 24 hours pulse labelled during with 15 μM BrdU, fixed and then analysed by FACS. **A-D** show the G_1-, S- and G_2-phase distribution for untreated cells (**A**); Peptide-I at 25μM (**B**); Peptide-II at 50 μM (**C**) and Peptide-III at 25 μM (**D**). Panel **E** shows the data from **A-D** represented as the % of cells in each phase compared to the total number of cells counted.

In a separate experiment (panel **F**) DMEM + 10% FCS alone or DMEM + 10% FCS containing either 25 μM Peptide-I or 50μM Peptide-II, was added to HaCaT cells than had been starved for 72 hours. Samples were taken at the times shown and analysed by SDS-PAGE/Western blot stained for pRb. pRb represents hypophosphorylated Rb protein and pRb* refers to hyperphosphorylated Rb protein. It should be pointed out that equal amounts of total protein were loaded per lane

and that the antibody appears to preferentially recognise phosphorylated forms of the Rb protein.

Experimental Procedures

Peptides

All peptides were synthesised by Chiron Mimotopes, Peptide Systems (Clayton, Australia). Each peptide had a Biotin-SGSG spacer at the C-terminus and a free N-terminus. The peptides were dissolved in DMSO at approximately 5 mg/ml and we then determined their concentration precisely by amino acid analysis (Smythe et al., 1988). In addition the purity of the peptides was estimated using mass spectrometry. Positive ion electrospray mass spectrometry was performed on a triple-quadruple mass spectrometer (V. G. Quattro) in (50/50/0.1) water/acetonitrile/formic acid.

Proteins

Cyclins and CDKs - Cdk4 and cyclin D1, and Cdc2 and cyclin B were co-expressed in Sf9 insect cells infected with the appropriate baculovirus constructs. The cells were harvested two days after infection by low speed centrifugation and the pellet was lysed in an equal volume of 10 mM Hepes, pH 7.4 containing: 10 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethane sulphonyl fluoride, 2 mM DTT and centrifuged at 14000 x g for 15 min. The supernatant was removed, aliquoted and immediately frozen in liquid nitrogen. Thawed lysate was used only once and was never refrozen. Labelled Cdk4 and cyclin D1 were produced by translation in the presence of [^{35}S] methionine using a rabbit reticulocyte lysate in vitro translation kit (Promega).

His-tagged $p21^{WAF1}$ - Human $p21^{WAF1}$ was expressed in E.coli using a PET expression vector. The soluble $p21^{WAF1}$ protein fraction was purified using a nickel chelating column,

following the manufacturers instructions (Pharmacia). The eluted protein peak was dialysed against 25 mM Hepes, pH 7.4, containing: 0.1 mM EDTA, 1 mM benzamidine, 0.01% Triton X-100, and 0.1 mM phenylmethane sulphonyl fluoride, concentrated and applied to a Superose 12 gel-filtration column (Phamacia) equilibrated in the above buffer. Fractions containing $p21^{WAF1}$ were detected by Western blot using the $p21^{WAF1}$ specific monoclonal antibody Ab-1 (Oncogene Sciences), concentrated to 200 μg/ml and stored at -70°C.

GST-Rb - An E. coli expression construct containing the hyperphosphorylation domain of pRb (amino acids 773-924) was purified on a glutathione-Sepharose column according to the manufacturers instructions (Pharmacia).

Peptide Precipitation of Cdk4 and Cyclin D1

A 20 amino acid peptide library, that spanned the entire sequence of $p21^{WAF1}$ (Figure 1), was screened for Cdk4/cyclin D1 interacting peptides. Peptide (1.5 μg) was diluted in 100 μl of PBS and incubated with 10 μl of packed streptavidin-agarose beads (Sigma) for 1 h at room temperature. Unbound peptide was removed by extensive washing with PBS and the beads, plus bound peptide, were incubated for 1h at 4°C with reticulocyte lysate containing either Cdk4 or cyclin D1 labelled with [^{35}S] methionine. The beads were washed three times with 1.25 x PBS containing 0.2% Triton X-100 and boiled in the presence of 0.125 M Tris-HCl, pH 6.8 containing: 4% (w/v) SDS, 20% (v/v) glycerol and 200 mM DTT. The bound protein was analysed by SDS-PAGE followed by auto-radiography and quantification of the ^{35}S-labelled protein using a Bio-Imager and Whole Band Analysis Software (Millipore).

Enzyme Assays

Phosphorylation of GST-Rb - Cdk4 activity was measured using the cyclin D1-Cdk4 containing insect cell lysate described above. Extract (1 μl) was added to a final

reaction volume of 10 μl, containing: 50 mM Hepes, pH 7.4, 10 mM MgCl2, 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, 10 mM PKI, 50 μM ATP containing [^{32}P] ATP (1 000 cpm/pMol) and 0.5 μg GST-Rb. The assays were started by the addition of the GST-Rb substrate, incubated at 30°C for 10 min (the incorporation of ^{32}P into GST-Rb was linear over 15-20 min) and terminated by adding SDS-PAGE sample buffer and heating at 95°C for 4 min. The samples were analysed by SDS-PAGE on 12% gels followed by auto-radiography and quantification using a Bio-Imager.

Peptide phosphorylation

The biotinylated peptides (1 μg) were incubated for 30 min at 30 °C in a final volume of 20 μl containing: 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, 10 mM PKI, 50 μM ATP containing [^{32}P]ATP (6000 cpm/pMol) and either 1 μl of cyclin D1-Cdk4 insect cell lysate, 1 μl of uninfected insect cell lysate or 0.02 mU of protein kinase C plus 0.5 mM $CaCl_2$, 100 mg/ml phosphatidyl serine and 20 mg/ml diacylglycerol. The reactions were stopped by heating at 60oC for 5 min and streptavidin agarose beads were added (10μl packed cell volume washed with 3 x PBS) and incubated with shaking at 4oC for 30 min. The beads were washed extensively with PBS containing 3% (v/v) Tween-20 and the incorporation of radioactivity into the peptides was determined by Cerenkov counting.

Cell Cycle Measurements

Carrier linked peptides were designed for delivery into proliferating HaCaT cells (see Figure 11). Cells were seeded on 30mm culture plates and grown to 50% confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS). Peptides were added to the medium and the cells were incubated for 24 hours. During the last 30 minutes of the incubation the

cells were pulse labelled in the presence of 15μM BrdU. The cells were trypsinised, fixed in absolute alcohol and prepared for FACS analysis using a single laser flow cytometer (Becton-Dickinson, FACScan) as previously described (Renzing et al, 1996).

pRb Phosphorylation in HaCaT Cells

HaCaT cells were seeded on 30mm culture plates at 25% confluency in DMEM with 10% FCS. The FCS was withdrawn after 24 hours and the cells were starved for 72 hours. At the end of this period the medium was supplemented with 10% FCS and carrier linked peptides. Samples were taken over a 24 hour time course and the cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) DOC, 0.1% (w/v) SDS,1 mM PMSF, 0.1 mg/ml aprotinin and 0.5 mg/ml leupeptin) for 30 minutes at 4°C. The phosphorylation statues of pRb was determined by Western blot analysis, as previously described (Fåhraeus et al, 1996) except that the blot was probed with a pRb polyclonal antibody (C-15, Santa Cruz).

Results

Peptide-Binding Assay for Cyclin D1 and Cdk4

Using a series of synthetic peptides that span the entire sequence of $p21^{WAF1}$ (Figure 1), we determined whether these peptides could mimic full length $p21^{WAF1}$ protein by forming a stable complex with either cyclin D1 or Cdk4. If peptide-binding mimetics of $p21^{WAF1}$ protein could be identified, then this would assist in identifying the minimal binding motif of $p21^{WAF1}$ protein required for cyclin D1-Cdk4 holoenzyme inhibition and whether $p21^{WAF1}$ was targeting the cyclin or the kinase subunit. This would also define a system for using small peptides to study $p21^{WAF1}$ protein reaction mechanism and to design mimetic drugs.

The peptide-binding assay involved quantifying the amount of ^{35}S-labelled cyclin D1 or Cdk4 which bound specifically to biotinylated peptides that were captured on streptavidin coated agarose beads. The peptide-coated beads were added to extracts containing either ^{35}S-labelled cyclin D1 or Cdk4 translated in vitro, the beads were washed extensively to remove unbound protein, and the bound cyclin D1 or Cdk4 was quantified by SDS-PAGE followed by auto-radiography and bio-imaging. This is referred to below as a peptide precipitation assay and has been used previously to demonstrate evolutionary conservation of $p21^{WAF1}$ binding to PCNA (Ball and Lane, 1996).

A Small Peptide Derived from Amino Acids 46-65 in the N-Terminal Domain of $p21^{WAF1}$ Binds Directly to Cdk4

Using the peptide-precipitation assay, peptide 4 (from the N-terminal domain of $p21^{WAF1}$) bound specifically to Cdk4, but not to cyclin D1 (Figure 1). This interaction is physiologically important, since the CDK interacting domain of the $p21^{WAF1}$ protein has previously been proposed to localise to the N-terminal domain of the molecule (Chen et al.,1995; Harper et al., 1995; Luo et al., 1995). More specifically, deletions (Nakanishi et al., 1995a) or mutations (Goubin and Ducommun, 1995) in the region of amino acids 45-71 compromise the ability of full length p21WAF1 to interact with Cdk2. Whether this loss of p21WAF1 binding function is due to, (i) mutation/deletion of residues directly involved in CDK binding, or (ii) mutations/deletion induced conformational alterations in $p21^{WAF1}$ that prevent stable binding to CDK, has not been demonstrated. Here we show unequivocally that residues 46-65 are directly involved in the binding of $p21^{WAF1}$ to Cdk4 and that alone they are capable of forming a stable complex with Cdk4, in the absence of cyclin D1. Thus, providing direct evidence that the N-terminus of $p21^{WAF1}$ does contain a kinase binding domain.

A Small Peptide Derived from Amino Acids 16-35 in the N-Terminus of p21^{WAF1} Binds Directly to Cyclin D1

We were also able to define a second and distinct N-terminal interaction site on the p21^{WAF1} protein; in this case a region of p21^{WAF1} which is capable of binding to cyclin D1, but not to Cdk4 (Figure 1). Peptide 2 comprises amino acids 16-35 of p21^{WAF1} and lies within the Èminimum region required for DNA synthesis inhibition in vivo, which is located between residues 17-71 (Nakanishi et al., 1995a). Our results might explain an apparent contradiction encountered by Nakanishi et al. (1995a) who found that N-terminal mutations in p21^{WAF1} protein which are outside the CDK interacting domain, although insufficient to prevent binding to the kinase, were sufficient to prevent p21^{WAF1} from acting as a growth suppressor when transfected into proliferating cells. Specifically, the direct peptide binding data (Figure 1) leads us to suggest that an N-terminal motif in the p21^{WAF1} protein, that mediates cyclin D1 binding, could be an essential step in the mechanism through which p21^{WAF1} protein functions as a growth suppressor.

A Novel Cyclin D1-Cdk4 Binding Motif Resides in the C-Terminus of the p21^{WAF1} Protein

The specificity of the peptide-precipitation assay in defining the domain of p21^{WAF1} protein required for binding to either the cyclin D1 or Cdk4 (Figure 1), indicated that using peptides to study potential interactions between p21^{WAF1} and cyclin-CDK complexes would prove to be very informative. We were intrigued however, by the finding that peptides from the C-terminus of the p21^{WAF1} protein (peptides 10 and 11) could form stable complexes with both Cdk4 and cyclin D1 (Figure 1), as peptide 10 is equivalent to the p21PBP peptide described by Warbrick et al. (1995) as representing the region of p21^{WAF1} which binds to the replication/repair protein PCNA. We can not rule out the

possibility that endogenous cyclin or CDK present in the reticulocyte lysate could bind to the labelled human protein forming a bridge to the peptide. However, as peptide 2 and peptide 4 precipitate either cyclin D1 or Cdk4, respectively, this seems unlikely. These results suggest that the $p21^{WAF1}$ protein may interact with both PCNA and cyclin-CDK complexes through the same binding motif. Peptide 11 however, binds to both Cdk4 and cyclin D1 but not to PCNA (Figure 1) (Warbrick et al., 1995; Ball and Lane, 1996); uncoupling the PCNA binding site from the cyclin/CDK binding motif in the C-terminus of $p21^{WAF1}$.

Given that we had identified three distinct motifs from the $p21^{WAF1}$ protein which bind specifically to cyclin D1 and/or Cdk4, we then examined whether they mimicked $p21^{WAF1}$ protein by inhibiting kinase activity.

The Cyclin D1 Binding Peptide from the N-Terminal Domain of $p21^{WAF1}$ and the Cyclin/CDK Binding Peptide from the C-Terminus of $p21^{WAF1}$ Inhibits the Activity of Cdk4

In order to determine if any of the $p21^{WAF1}$ peptides possessed Cdk4 inhibitory activity we tested, independently, their ability to prevent pRb phosphorylation during cyclin D1-Cdk4 assays in vitro (Figure 2). Peptides 2, 8,10, and 11 inhibited cyclin D1-Cdk4 activity when added to the assay at 17 μM, whereas buffer alone and the remaining peptides had no dramatic affect on Cdk4 activity. The cyclin D1 binding peptide (peptide 2) inhibited the kinase activity by approximately 80% and peptides 10 and 11, which bound both Cdk4 and cyclin D1, completely inhibited enzyme activity at this concentration. Thus, there is a correlation between the ability of the peptides to bind to Cdk4 and/or cyclin D1 and to inhibit Cdk 4 kinase activity.

However, this correlation breaks down in the case of kinase-binding peptide 4. This peptide maps to the CDK

interaction site (Figure 10; Goubin and Ducommun, 1995; Nakanishi et al., 1995a) and there has been speculation that a peptide from this domain, capable of interacting with CDK, would mimic full-length $p21^{WAF1}$ inhibitory activity, and would therefore provide a model for the design of novel molecules that could arrest cell cycle progression by inhibiting the G1 cyclin-CDKs. Although of high affinity for Cdk4, peptide 4 had no inhibitory activity when added to cyclin D1-Cdk4 assays at concentrations of up to 35 μM. Our data from both $p21^{WAF1}$-peptide binding data and inhibitory properties, therefore pinpoints two novel small domains of the $p21^{WAF1}$ protein as potential candidates for small molecular weight mimetics; an N-terminal motif from amino acids 16-35 (peptide 2) and a C-terminal motif from amino acids 141-160 (peptide 10).

The C-Terminal $p21^{WAF1}$ Peptide is a More Potent Inhibitor of Cdk4 Kinase Activity than the N-Terminal Cyclin D1-Binding Peptide

We carried out more detailed studies to determine the Ki for peptides 2, 8, and 10, using peptide 4 as a negative control (Figure 3). We found that peptide 10 (and peptide 11; data not shown) was a potent inhibitor of Cdk4 activity with a Ki of 0.1 μM, peptide 2, was also a good inhibitor with a Ki of 2 μM. Peptide 8 gave only weak inhibition and relatively high concentrations of peptide were required to approach 50% inhibition. These data support the possibility of using peptide 2 or peptide 10 to mimic the CDK inhibitory activity of the full length $p21^{WAF1}$ protein.

$p21^{WAF1}$ Protein and Inhibitory Peptides Compete for the Same Binding Site on Cdk4 Kinase

In order to determine if the Cdk4 inhibitory peptides, 2 and 10, were acting at sites on Cdk4 and cyclin D1 that were also employed by $p21^{WAF1}$, we carried out peptide precipitation assays in the presence and absence of full

length purified his-$p21^{WAF1}$ to find out if it competed with the peptides for binding (Figure 4, top panel). The data suggest that binding of $p21^{WAF1}$ protein to cyclin D1 and Cdk4 prevents binding of both peptide 2 and peptide 10. These data are open to two interpretations, (i) the peptides could be competing for binding at the same site as $p21^{WAF1}$, or (ii) binding of either $p21^{WAF1}$ or peptide could cause a conformational change in the cyclin or CDK preventing further binding. It is not clear from these experiments whether peptides 2 and 10 are acting at the same site(s). However the difference in the peptide precipitation data, indicates that at least one of the sites is unique, as peptide 10 can precipitate both Cdk4 and cyclin D1, whereas, peptide 2 can only precipitate cyclin D1.

Data to support the hypothesis that peptide 10 and $p21^{WAF1}$ protein compete for the same binding site, during kinase inhibition, employs the use of a peptide 10 mutant (containing a point mutation resulting in a change of R - A at residue 15 of peptide 10 which is equivalent to residue 155 of the full length protein) which loses >60% of its inhibitory activity (see below), but retains its binding function. The experiment showed that increasing concentrations of mutant peptide 10 were able to block the inhibitory activity of full length $p21^{WAF1}$ (Figure 4, bottom panel), suggesting that peptide 10 is binding at a site(s) which blocks subsequent binding of $p21^{WAF1}$ and is therefore functioning through a similar mechanism to the full length protein.

The Inhibitory Peptides are not Cyclin D1-Cdk4 Substrates

Unlike the p107 protein, which appears to inhibit Cdk4s ability to phosphorylate pRb by acting as an alternative substrate (Zhu et al., 1995), $p21^{WAF1}$ has not been reported to act as a substrate for the cyclin D1-Cdk4 complexes (and we confirm these observations Figure 5). However, it is possible that by using $p21^{WAF1}$ based peptides, instead of

full length protein, we have inadvertently generated phosphorylation sites which would not normally be exposed on the surface of the protein. Thus the peptides could be acting as competitive substrates as opposed to inhibitors of catalytic activity. Both peptide 2 and peptide 10 contain a number of possible phosphorylation sites, and we have been able to demonstrate that peptide 10 is a potential substrate for a number of protein kinases (data not shown), including protein kinase C (PKC) which was used as a control kinase (Figure 5). In fact, neither peptide 2 nor peptide 10 were substrates for cyclin D1-Cdk4 under conditions where 2.4 nMol of ^{32}P were incorporated per nMol of GST-Rb. However, under the same conditions peptide 10 was an extremely good substrate for PKC with 0.82 nMol of ^{32}P being incorporated per nMol of peptide (Figure 5). There was a low level of incorporation into peptide 2, but as this was also present in assays using lysate from uninfected insect cells it must be attributed to low levels of endogenous protein kinase(s). Thus, it appears that the peptide inhibitors are not competitive substrates, but, are acting to block catalytic activity in a mechanism similar to $p21^{WAF1}$.

The Peptides are not Efficient Inhibitors of Cyclin B-Cdc2 Kinase Activity

Harper et al. (1995) have shown that $p21^{WAF1}$ is not a universal CDK inhibitor, but that it displays selectivity for the G1 and S-phase cyclin-CDK complexes. When they compared the ability of $p21^{WAF1}$ to inhibit Cyclin B-Cdc2, which acts at the G2/M transition, and cyclin D2-Cdk4, which functions during G1, they found that the Ki for inhibition of cyclin B-Cdc2 was > 600-fold higher than the Ki for inhibition of cyclin D2-Cdk4 using purified recombinant proteins. We looked at the effect of adding our two cyclin D1-Cdk4 inhibitory peptides to cyclin B-Cdc2 assays at concentrations up to 20 μM and found that neither peptide 2 nor peptide 10 had a significant effect on the

enzymes histone H1 kinase activity (Figure 6). Thus, the $p21^{WAF1}$ based peptide inhibitors appear to have equivalent specificity to the full length protein with regard to cyclin B-Cdc2.

The Kinase Inhibitory Motif of Peptide 10 is Distinct from the PCNA Binding Site

We have shown that peptide 10 is an extremely potent inhibitor of cyclin D1-Cdk4 activity, with a Ki of 0.1 mM which is 20-fold more potent than peptide 2, a peptide derived from the region of $p21^{WAF1}$ previously associated with growth arrest (Chen et al.,1995; Nakanishi et al., 1995a). We have also shown that a peptide (peptide 4) which spans the CDK interaction site of $p21^{WAF1}$ (Goubin and Ducommun, 1995; Nakanishi et al., 1995a), although capable of binding to Cdk4 to form a stable complex, has no detectable activity as a cyclin D1-Cdk4 inhibitor. Peptide 10 therefore looks like the best candidate for the development of a small peptide mimetic with high efficacy. Peptide 10 has previously been shown to form a specific high-affinity and reversible interaction with PCNA (Ball and Lane, 1996) and this peptide is sufficient to partially inhibit the function of PCNA during SV40 replication giving 50% inhibition at a concentration of approximately 7 mM (Warbrick et al., 1995). The PCNA interaction domain of $p21^{WAF1}$ has been mapped and the important residues were found to be amino acids 144-151 (QTSMTDFY; Warbrick et al., 1995; Ball and Lane, 1996). Although the extreme C-terminal peptide (peptide 11) has amino acid residues important for binding to and inhibiting Cdk4 (see Figure 1 and 2), it cannot bind PCNA (Warbrick et al., 1995; Ball and Lane, 1996). These results indicate that the kinase inhibitory and PCNA binding motif in the C-terminus of $p21^{WAF1}$ are distinct, but it does not rule out the possibility that an interaction between $p21^{WAF1}$ and PCNA or cyclin/kinase may require some common amino acids. It is therefore important to identify the precise inhibitory motif within the

C-terminus of $p21^{WAF1}$ and establish if it overlaps, or is distinct from, the PCNA interaction domain. To investigate this question we took two approaches; we synthesised, (i) a series of peptides that had been shifted by 4 amino acids in either direction along peptide 10 (size scan; Figure 7), and (ii) a series of peptides based on peptide 10 where each residue was sequentially mutated to alanine (alanine scan; Figure 8). The ability of the peptides, in each of these two series, to inhibit Cdk4 activity in vitro was then determined. Using the size scan, we found that the peptide inhibition activity required amino acids 156-160, while amino acids 148-155 were dispensable (Figure 7). This uncouples the kinase inhibitory motif from the PCNA binding motif.

With the alanine scan we defined the critical residues for inhibition showing that a stretch of just 5 amino acids were essential for activity, with a single conservative point mutation at either of two hydrophobic residues completely abolishing peptide 10 inhibitory activity (Figure 8). The essential amino acids are RRLIF (amino acids 155-160) where the bold characters are essential for activity and the underlined residue contributes significantly to inhibitory activity.

When tested in the peptide precipitation assay, mutation of the first R of this motif to A (aa 155 of full length $p21^{WAF1}$) partially retained its ability to bind both Cdk4 and cyclin D1, whereas mutations of L or F to A significantly decreased the affinity for Cdk4 and cyclin D1, and mutations of the second R or the I had no effect on binding (data not shown). This is why the R - A mutant was used in competition assays (Figure 4). The fact that a single point mutation in either of two hydrophobic residues (the L or F residues) completely abolishes inhibitory activity, suggested that inhibition was due to a specific interaction at key hydrophobic residues. The mapping data also

explains why both peptide 10 and peptide 11 are good inhibitors of cyclin D1-Cdk4 activity (Figure 2) as they both contain the inhibitory motif. Thus, it appears that the inhibitory portion of peptide 10 does not overlap with the PCNA binding site as they have no amino acid residues in common.

A Single Amino Acid Substitution in Peptide 10 Makes it a More Potent Inhibitor thus Approaching the Specific Activity of Full Length p21^{WAF1} Protein

Whilst carrying out the alanine scan experiments we noticed that one of the mutant peptides (D - A at position 9 of peptide 10 or 149 of the full length protein; Figure 8) appeared to make the peptide a better inhibitor of cyclin D1-Cdk4 activity. We determined the Ki for this peptide and compared it with peptide 10, full length purified his-p21^{WAF1}, and a peptide derived from the tumour suppressor protein p16 INK4 which has recently been reported to inhibit cyclin D1-Cdk4 activity in vitro and to prevent cell cycle progression (Fahraeus et al, 1996). The D-A mutation decreases the Ki from 100 nM to 46 nM (Figure 9). Comparing this with the p16INK4-based peptide, which has a Ki of 16.3 μM (Figure 9), we have now produced a peptide which is approximately 350-fold more active as a Cdk4 inhibitory compound. In fact, we now begin to approach the potency of p21^{WAF1} itself, which has a Ki of 11 nM in the insect cell lysate assay (Figure 9). This value is in the same range as the Ki of 40 nM for p21^{WAF1} obtained for the inhibition of cyclin D1-Cdk4 in Sf9 cell lysates by Harper et al. (1995). Compared to full length protein, the mutant peptide 10 has only a 3.5-fold lower specific activity as a kinase inhibitor in crude lysates. Why mutating the D - A in this position, which is well out side the domain shown to be essential for activity, reduces the Ki is not known. It seems likely that it involves the presentation of the inhibitory motif, rather than a direct role for this residue in inhibition, as this mutation does not appear to

increase the affinity of the peptide for either Cdk4 or cyclin D1 (data not shown).

The results indicate that peptide 10 could be used as a model on which to base small peptide mimetics of $p21^{WAF1}$ and we have provided evidence that alterations in the peptides structure or presentation of the active residues may lead to the generation of a peptide inhibitor which approaches the potency of full length $p21^{WAF1}$ as a cyclin D-Cdk4 inhibitor.

<u>Results for the C-terminal peptide (peptide 10)</u>

A Seven Amino Acid Peptide is Sufficient to Inhibit Cyclin D-Cdk4 Activity

Having identified residues which appeared to be critical for the inhibition of cyclin D1-Cdk4 by peptide 10, we determined if these residues were sufficient for inhibition, or if they had to be presented within the context of a larger peptide. Strikingly, the eight amino acid peptide, KRRLIFSK, retained the ability to completely inhibit cyclin D1-Cdk4 activity and prevent phosphorylation of pRb (Figure 8). However, the K_i for the truncated peptide was approximately 1000-fold higher than that of the full length peptide (K_i for the truncated peptide was approximately 100 μM). This was not an unexpected result as other studies have shown loss of potency upon reducing the length of bio-active peptides. However, it may be possible to improve the peptide inhibitory activity by manipulating the non-essential residues in a manner defined by Lin et al (1995) in an elegant series of experiments aimed at minimising the atrial natriuretic peptide.

Peptide 10 works in cell systems

The introduction of $p21^{WAF1}$ cDNA into human brain, lung and colon cancer cell lines leads to a suppression of cell growth (El-Deiry et al, 1993). In addition, during a radiation-induced G_1 arrest in human fibroblasts $p21^{WAF1}$

protein levels increase, in a p53-dependent manner, leading to potent inhibition of the G_1 cyclin-CDKs and failure of the cells to enter S-phase (Dulic et al, 1994; Harper et al, 1995). In order for peptide 10 to function as a realistic template for the design of novel anti-proliferative drugs it must be able to mimic $p21^{WAF1}$'s CKI activity as a growth suppressor in a cellular background. We and others have recently shown that a 16 amino acid sequence from the homeodomain of the Antennapedia protein can act as a carrier for peptides with biological activity, translocating them across the plasma membrane and allowing them to interact with their target molecules (Fåhraeus et al, 1996; Hall et al, 1996). To determine if peptide 10 retained its biological activity when introduced into tissue culture cells, we synthesised it directly onto the carrier peptide and added it to a culture of proliferating asynchronous human kerotinocyte-derived HaCaT cells. The linked peptide (designated Peptide-I; Figure 11) contained a mutation of M to A at position 7, thus abolishing its activity as a PCNA binding peptide (Warbrick et al, 1995; Ball and Lane, 1996), and allowed us to study PCNA-independent affects of the peptide on normal cell cycle.

Peptide-I was added to the culture media at a concentration of 25 μM, the cells were fixed 24 hours later, and then analysed by fluorescence-activated cell sorting (FACS). Figure 11 (A and B) shows the G_1-, S- and $_2$ G -phase distribution of untreated and Peptide-I treated cells assayed using bromodeoxyuracil (BrdU). The number of cells entering S-phase in the presence of Peptide-I was dramatically reduced and the G_1 population showed a concomitant increase. This suggests that Peptide-I mimics the ability of full length $p21^{WAF1}$ to act as a growth suppressor by inducing a G_1-cell cycle arrest.

In order to ascertain if Peptide-I was functioning as a growth inhibitor by preventing the phosphorylation of pRb

in a manner analogues to $p21^{WAF1}$, we used serum starvation to produce a synchronous population of HaCaT cells. Peptide-I was added to the cells at the same time as they were released from serum starvation and samples from treated and untreated cells were taken over a 24 hour period. The phosphorylation status of pRb was monitored by a gel mobility shift assay (Figure 10-F). When serum was added to starved cells, pRb became hyperphosphylated between 12 and 15 hours, but in the presence of Peptide-I pRb remained hypophosphorylated. Thus, Peptide I causes a G_1-arrest in human HaCaT cells by preventing the phosphorylation of pRb.

We took an identical experimental approach to introduce, (i) the bio-active truncated peptide 10 (Figure 11-C; Peptide-II), and (ii) a control peptide 10 which lacked essential residues for CDK inhibition (Figure 11-D; Peptide-III), into HaCaT cells. We found that Peptide-II effectively promoted a G_1-phase arrest and totally prevented the phosphorylation of pRb when added at 50μM (Figure 11-F). However Peptide-III, which lacked the last 4 amino acids of peptide 10 (LIFS) had no detectable effect on the ability of HaCaT cells to enter S-phase. It is interesting that the truncated peptide 10 when coupled to carrier peptide (Peptide-II) and introduced into cells is only 2-fold less active as a growth suppressor than Peptide-I (see above for in vitro data). Linking the truncated peptide 10 to the carrier peptide may promote a more favourable inhibitory conformation, as the K_i for carrier linked truncated peptide 10 (Peptide-II in vitro is approximately 50-fold less than that of the free peptide 10 (data not shown).

Discussion

Synthetic peptides or peptido-mimetics are proving to be useful in studying the biochemical regulation of enzymes and proteins, and also in providing models for the design

of novel anti-proliferative agents targeted to the enzymatic pathways amplified or proteins activated in human tumours (Powis, 1992; Gibbs and Oliff, 1994). Peptides which have been shown to effectively target components of the cell cycle machinery include: FTI, which inhibit farnesyl protein transferase preventing the activation of Ras (Gibbs et al., 1994); Ras effector domain peptides, which can inhibit its biological function (Moodie and Wolfman, 1994; Rodriguez-Viciana et al., 1994); SH2/SH3 domain-harbouring polypeptides, which in theory should inhibit the growth of tumours with activated tyrosine kinases (Pawson and Schlessinger, 1993; Yu et al., 1994), and p16INK4-derived peptides, which inhibit cyclin D-CDK complex activity and thereby activate pRb-dependent cell cycle arrest (Fåhraeus et al., 1996).

Inactivation of the tumour suppressor protein p53 is a common event in the development of human neoplasia (Hollstein et al., 1991). The p53 protein is a key player in an inducible cell cycle checkpoint pathway activated in response to DNA-damage and nucleotide pool perturbation (Lane, 1992; Agarwal et al., 1995). Reactivation of this pathway could therefore provide a route to the discovery of novel anti-proliferative drugs. A variety of mechanisms could lead to the functional inactivation of the p53 pathway, including the inactivation of downstream effector molecules of p53, such as the cyclin-kinase inhibitor p21WAF1 (Deng et al., 1995; Waldman et al., 1995). Recent developments have shown that reactivation of the p53 pathway in some human tumours may be possible by activating the biochemical function of the endogenous mutant p53 protein (Halazonetis and Kandil, 1993; Hupp et al., 1993), possibly using small peptides as leads for drug design (Hupp et al., 1995) or by reintroducing the wild type p53 gene using adenovirus vectors (Eastham et al., 1995). However, in general, the pharmacological restoration of biochemical function to a protein that has lost its normal

activity through mutation of its amino acid sequence is more difficult than the inhibition of a biochemical function (Gibbs and Oliff, 1994). Thus, it may prove more productive to take alternative approaches to restore activity to the p53 pathway such as mimicking the inhibitory activity of the downstream effector molecule p21WAF1, which can by itself mediate growth arrest primarily through its interaction with the G1 cyclin-CDKs (El-Deiry et al., 1993; Eastham et al., 1995; Harper et al., 1995).

Determining the minimal domain of $p21^{WAF1}$ that can inhibit CDK function and whether such a domain can function in isolation with high efficiency are two important goals which must be achieved in order to determine whether $p21^{WAF1}$ will prove to be a realistic template for use in anti-proliferative drug design research. Prior to our studies, the minimal sequence of $p21^{WAF1}$ shown to inhibit CDK function in vitro was the N-terminal domain (residues 1-75) (Luo et al., 1995). Whilst peptides derived from this N-terminal domain have recently been shown to antagonise the ability of $p21^{WAF1}$ to inhibit cyclin E-Cdk2 complex activity suggesting that this domain interacts with the kinase (Chen et al., 1996), no data on the direct interaction of small peptides with either cyclin or CDK has previously been presented. In addition, no evidence existed to suggest that a small peptide derived from $p21^{WAF1}$ would in fact be biologically active as a CDK inhibitor. As the cyclin D1-Cdk4 complexes and related isoforms are essential for progression through G1-phase, we have used a series of small synthetic peptides based on the sequence of p21WAF1 to, (i) determine whether Cdk4 inhibitory peptide-mimetics exist and if they are of high efficacy, and (ii) probe the mechanism by which the p21WAF1 protein inhibits cyclin D1-Cdk4 activity.

A Model for the Allosteric Inhibition of Cyclin D1-Cdk4 by $p21^{WAF1}$

Two distinct peptides from the N-terminal domain of $p21^{WAF1}$ interacted with either Cdk4 or cyclin D1 to form stable complexes. One peptide bound to Cdk4 but did not inhibit its activity, while the second bound specifically to cyclin D1 and had potent inhibitory effects on cyclin D1-Cdk4 activity. The Cdk4 binding peptide 4 (residues 46-65) corresponded to a putative Cdk2 binding domain of $p21^{WAF1}$ previously defined using $p21^{WAF1}$ deletion constructs (Nakanishi et al., 1995a) and alanine mutation analysis (Goubin and Ducommun, 1995). We have established that this region of $p21^{WAF1}$ is, in fact, directly involved in CDK binding, yet it has no Cdk4 inhibitory activity (Figures 2 and 3). These data explain why certain N-terminally deleted $p21^{WAF1}$ constructs, which still contain the CDK binding site, fail to efficiently inhibit cell growth (Nakanishi et al., 1995a).

The second N-terminal peptide, which bound to cyclin D1, potently inhibited cyclin D1-Cdk4 activity through a novel mechanism (see below). The mechanism of $p21^{WAF1}$ inhibition of cyclin-CDK complexes is poorly understood, as it has not been clear whether $p21^{WAF1}$ protein inhibits by cyclin and/or kinase subunit binding. Cdk2 binds very weakly to p21WAF1 in the absence of cyclin, the affinity of the G1-CDKs for $p21^{WAF1}$ being greatly increased if the CDK is associated with a cyclin (Harper et al., 1995), suggesting that cyclins play an important role in $p21^{WAF1}$ inhibition of CDK activity. However, whether a CKI, such as $p21^{WAF1}$ and p27KIP1, can interact directly with cyclin is in dispute (Toyoshima and Hunter, 1994; Harper et al., 1995). A recent study however, suggested that $p21^{WAF1}$ can interact directly with a number of cyclins in the absence of CDK (Fotedar et al., 1996). We show here that a small peptide composed of residues 16-35 (peptide 2) forms a stable complex with cyclin D1 and that this peptide alone is a potent inhibitor

of Cdk4 activity, with a Ki of 2 mM. This peptide falls within the growth suppressor region (residues 17-71), described by Nakanishi et al. (1995a). This is the first time that a putative cyclin binding site on $p21^{WAF1}$ has been identified and that a small synthetic peptide representing this domain has been shown to be sufficient to mimic the full length $p21^{WAF1}$ protein as a CDK inhibitor.

The fact that cyclin D1-Cdk4 activity can be inhibited by interaction with the cyclin subunit alone, suggests that conformational changes in cyclin D1 can lead to the inhibition of Cdk4 catalytic activity. Thus, we propose that one mechanism by which $p21^{WAF1}$ can affect Cdk4 activity is by allosteric inhibition mediated through conformational changes in the cyclin subunit. In addition, our results may offer an explanation of how active and inactive $p21^{WAF1}$ containing cyclin-CDK complexes can exist and how the loading of multiple $p21^{WAF1}$ molecules onto cyclin-CDKs can cooperatively inhibit kinase activity (Zhang et al., 1994; Harper et al., 1995).

In our model of $p21^{WAF1}$ inhibition of cyclin D1-Cdk4 kinase function (Figure 10), we propose that one molecule of $p21^{WAF1}$ preferentially binds first to Cdk4 through its CDK interacting site (residues 46-65) without affecting kinase catalytic activity. This is consistent with previous reports that cyclin-CDK complex associated with a single p21WAF1 molecule are catalytically active (Zhang et al., 1994). After a second molecule of $p21^{WAF1}$ binds to the cyclin D1 subunit, through interaction with $p21^{WAF1}$ residues 16-35, allosteric inhibition of kinase function is achieved. It is also possible that the rate of binding of the second molecule of $p21^{WAF1}$ to the cyclin D1 subunit could be facilitated by the presence of the first at the kinase subunit, leading to cooperative inhibition.

Prospects for the design of small molecular mimetics of $p21^{WAF1}$ are more viable given that the cyclin D1-binding peptide alone can inhibit kinase function, indicating that the prior presence of one $p21^{WAF1}$ protein binding to the kinase subunit is not required for allosteric inhibition of kinase function. In addition, the amino acid residues that are conserved between $p21^{WAF1}$ and its close relative p27KIP1 (Polyak et al., 1994; Toyoshima and Hunter, 1994) are clustered within the N-terminal domain, with the regions corresponding to peptides 2 (65% identical) and peptide 4 (50% identical) containing the majority of the conserved amino acids. This suggests that allosteric inhibition of Cdk4 activity by interaction with the cyclin D subunit may be a common mechanism employed by both $p21^{WAF1}$ and p27KIP1.

A Novel $p21^{WAF1}$ C-Terminal Cyclin D1-Cdk4 Inhibitory Domain

During the course of our studies we also found that a peptide (peptide 10) from the C-terminal domain of $p21^{WAF1}$ was a potent inhibitor of cyclin D1-Cdk4 activity in vitro. The inhibitory motif was identified and was distinct from the PCNA interacting site, which also resides in the C-terminal domain of $p21^{WAF1}$ (Chen et al., 1995; Luo et al., 1995; Warbrick et al., 1995; Ball and Lane, 1996). Our results are in contrast to previous studies which have found that cyclin-Cdk2 inhibitory activity is confined solely to the N-terminal domain of $p21^{WAF1}$, when each half is expressed separately (Chen et al., 1995; Luo et al., 1995). The reasons for this discrepancy may include: (i) the use of C-terminally his-tagged $p21^{WAF1}$ in expression vectors for purifying $p21^{WAF1}$ constructs (Luo et al., 1995), which may have affected the local structure at the C-terminus of $p21^{WAF1}$; (ii) the transfection of constructs containing only the C-terminal half of $p21^{WAF1}$ (Chen et al., 1995; Luo et al., 1995) this may make folding into the correct native conformation difficult precluding identification of the novel inhibitory domain; (iii) by using peptides, rather

that the C-terminal constructs or full length $p21^{WAF1}$ protein, we may have exposed sites which would not be solvent exposed in native full length $p21^{WAF1}$ protein; (iv) it is possible that there may be subtle differences in the mechanism(s) used by $p21^{WAF1}$ to inhibit cyclin-Cdk2 complexes and cyclin D1-Cdk4. Whether, the C-terminal inhibitory motif defines a novel physiologically relevant regulatory site on $p21^{WAF1}$ is currently being addressed. However, the potency of peptide 10 (Ki =0.1 mM, only 10-fold lower than full length $p21^{WAF1}$ protein in these assays) and its ability to completely inhibit cyclin D1-Cdk4, suggests to us that further studies on this region of full length $p21^{WAF1}$ will be well worth pursuing.

Peptide 10 represents a potentially exciting lead for drug design as it is by far the most potent peptide inhibitor of CDK activity discovered to date, being > 150-fold better than the recently identified peptide mimetic of p16INK4 (Fahraeus et al., 1996) and 20-fold better than the N-terminal inhibitory $p21^{WAF1}$-derived-peptide which we have described. The fact that the residues important for inhibitory activity are confined to a stretch of just five amino acids, suggests that contact at a single interface is sufficient to produce a highly potent inhibitor of the cyclin D1-Cdk4 activity, making this a realistic template for the design of small molecules which mimic $p21^{WAF1}$ activity.

The fact that peptide 10 retains inhibitory activity when reduced to just eight amino acids (KRRLIFSK) improves its appeal as a template for rational drug design. In general protein-protein interfaces are relatively large relying on the participation of between 10-30 contact side chains on each interface, with each region of contact often being composed of residues which are dispersed throughout the primary amino acid sequence (Davies et al, 1990; de Vos et al, 1992). However, there is evidence that in some cases

only a small subset of these side chains need to be contacted for efficient binding to occur (Kelley and O'connel, 1993, Cunningham and Wells, 1994; Clackson and Wells, 1995). The discovery that a single eight amino acid peptide is alone sufficient to inhibit the activity of a critical G_1-cyclin-CDK preventing pRb phosphorylation and producing a G_1-cell cycle arrest in tissue culture cell systems, suggests that interaction at only a small subset of contact side chains is necessary for potent inhibition of cyclin D1-Cdk4 activity at the G1-S phase boundary. This makes cyclin D1-Cdk4 a realistic and exciting target for the design of small synthetic compounds which can at act as anti-proliferative agents.

References

The following references are hereby incorporated by reference.

Agarwal, M., Agarwal, A., Taylor, W., and Stark, G. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 92, 8493-8497.

Baldin, V., Lukas, J., Marcote, M. J., Pagano, M. and Draetta (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. 7, 812-821.

Ball, K.L. and Lane, D.P. (1996). Human and plant PCNA proliferating cell nuclear antigen share a highly conserved binding specificity for the p53 inducible gene product $p21^{WAF1}$. European J. Biochem. 237, 854-861.

Buckbinder, L., Talbot, R., Velasco-Miguel, S., Takenaka, I., Faha, B., Siezinger, B. R. and Kley, N. (1995). Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature 377, 646-649.

Chen, I. T., Akamatsu, M., Smith, M.L., Lung, F-D. T., Duba, D., Roller, P. P., Fornace Jr, A. J. and O'Connor, P. M. (1996). Characterization of p21Cip1/Waf1 peptide domains required for cyclin E/Cdk2 and PCNA interaction. Oncogene 12, 595-607.

Chen, J., Jackson, P. K., Kirschner, M. W. and Dutta, A. (1995). Seperate domains of p21 involved in the inhibition of Cdk and PCNA. Nature 374, 386-388.

Clackson, T. and Wells J.A. (1995). A hot spot of binding energy in a hormone-receptor interface. Science 267, 383-386.

Clarke A.R., Purdie C.A., Harrison D.J., Morris R.G., Bird C.C., Hooper M.L., and Wyllie A.H.(1993). Thymocyte apoptosis induced by p53 dependent and independent pathways. Nature 362, 849-852.

Cunningham, B.C. and Wells, J.A. (1994). Comparison of a structural and functional epitope. J. Mol. Biol. 234, 554-563.

Davies, D.R., Padlan, E.A. and Sheriff, S. (1990). Antibody-antigen complexes. Annu. Rev. Biochem. 59, 439-473.

Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell 82, 675-684.

de Vos, A.M., Ultsch, M. and Kossiakoff, A.A. (1992). Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 255, 306-312.

Dulic, V., Kaufmann, W.K., Wilson, S.J., Tisty T.D., Lees, E., Harper, J.W., Elledge, S.J. and Reed, S.I. (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 76, 1013-1023.

Eastham, J. A, Hall, S. J., Sehgal, I. et al. (1995). In vivo gene therapy with p53 or p21 adenovirus for prostate cancer. Cancer Res. 55, 5151-5155.

El-Deiry, W. S., Tokino, T., Velculescu, V. E., et al. (1993). WAF-1, a potential mediator of p53 tumor suppression. Cell 75, 817-825.

Fahraeus, R., Paramio, J. M., Ball, K. L., Lain, S. and Lane D. P. (1996). Inhibition of pRb phosphorylation and cell cycle progression by a 20 aa synthetic peptide derived from p16CDKN2. Curr. Biol. 6, 84-91.

Flores-Rozas, H., Kelman, Z., Dean, F. B., Flores- Rozas, H., Kelman, Z., Dean, F.B., Pan, Z.-Q., Harper, J. W., Elledge, S. J., OkDonnell, M. and Hurwitz, J. (1994). Cdk-interacting protein 1 binds with proliferating cell nuclear antigen and inhibits DNA replication catalysed by the DNA polymerase d holoenzyme. Proc. Natl. Acad. Sci. U.S.A. 91, 8655-8659.

Gibbs, J.B. and Oliff, A. (1994). Pharmaceutical research in molecular oncology. Cell 79,193-198.

Gibbs, J.B., Oliff, A. and Kohl, N.E. (1994). Farnesyltransferase inhibitors: Ras research yields a potential cancer therapeutic. Cell 77, 175-178.

Goubin, F. and Ducommun, B. (1995). Identification of binding domains on the p21Cip1 cyclin-dependent kinase inhibitor. Oncogene 10, 2281-2287.

Gu. Y, Turck, C.T., and Morgan, D. O. (1993). Inhibition of Cdk2 activity in vivo by an associated 20K regulatory subunit. Nature 371, 257-261.

Halazonetis, T.D. and Kandil, A.N. (1993). Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. EMBO J. 12, 5057-5064.

Hall, H., Williams, E.J., Moore, S.E., Walsh, F.S., Prochiantz, A. and Doherty, P. (1996). Inhibition of FGF-stimulated phosphatidylinositol hydrolysis and neurite outgrowth by a cell-membrane permeable phosphopeptide. Curr. Biol. 6, 580-587.

Harper, J. W., Adami, G. R., Wei, N., Keyomasi, K. and Elledge, S. J. (1993). The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75,805-816.

Harper, J. W., Elledge, S. J. Keyomasi, K., Dynlacht, B., Tsai, L-H., Zhang, P., Dobrowolski, C. B., Connell-Crowley, L., Swindell, E., Fox, M. P. and Wei, N.(1995). Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell 6, 387-400.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. (1991). p53 mutations in human cancer. Science 253, 49-53.

Hupp, T. R., Meek, D. M., Midgley, C. A. and Lane, D. P. (1993). Activation of the cryptic DNA binding function of mutant forms of p53. Nucl. Acids Res. 21, 3167-3174.

Hupp, T.R., Sparks, A., and Lane, D.P. (1995). Small peptides activate the latent sequence-specific DNA binding function of p53. Cell 83, 337-345.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51, 6304-6311.

Kastan, M. B., Zhan, Q., El-Deiry, W. K., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B.S., Vogelstein, B. and Fornace, A. J. (1992). A mammalian cell cycle checkpoint pathway utilising p53 and gadd45 is defective in Ataxia-Telangiectasia. Cell 71, 587-597.

Kearsey, J. M., Shivji, M. K. K., Hall, P A. and Wood, R. D. (1995). Does the p53 up regulated Gadd45 protein have a role in excision repair. Science 270, 1004-1005.

Kelley, R.F. and O'Connell, M.P. (1993). Thermodynamic analysis of an antibody functional epitope. Biochemistry 32, 6828-6835.

Lane, D. P. (1992). p53, guardian of the genome. Nature 358, 15-16.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A. and Jacks, T. (1993). p53 is required for radiation induced apoptosis in mouse thymocytes. Nature, 362, 847-849.

Luo, Y., Hurwitz, J. and Massague, J. (1995). Cell-cycle inhibition by independent CDK and PCNA binding domains in p21WAF1. Nature 375, 159-161.

Lu, X. and Lane, D.P. (1993). Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes. Cell 75,765-778.

Merritt, A.J., Potten, C.S., Kemp, C.J., Hickman, J.A., Balmain, A., Lane, D.P. and Hall,P.A. (1994). The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. Cancer Res. 54, 614-617.

Miyashita, T. and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80 , 293-299.

Momand, J., Zambetti, G. P., Olson, D. C. and Levine, A. J. (1992). The mdm-2 oncogene product forms a complex with p53 protein and inhibits p53-mediated transactivation. Cell 69, 1237-1245.

Moodie, S.A. and Wolfman, A. (1994). The 3Rks of life: Ras, Raf, and growth regulation. Trends Genet. 10, 44-48.

Nakanishi, M, Robetorye, R. S., Adami, G. R., Pereira-Smith, O. and Smith, J. (1995a). Identification of the active region of the DNA synthesis inhibitory gene p21Sdi1/CIP1/WAF1. EMBO J. 14, 555-563.

Nakanishi, M, Robetorye, R. S., Pereira-Smith, O. and Smith, J. (1995b). The C-terminal region of p21SDI1/WAF1/CIP1 is involved in proliferating cell nuclear antigen binding but does not appear to be required for growth inhibition. J. Biol. Chem. 270, 17060-17063.

Pawson, T. and Schlessinger, J. (1993). SH2 and SH3 domains. Current Biology 3, 434-432.

Picksley, S.M., Vojtesek, B., Sparks, A. and Lane, D.P. (1994). Immunochemical analysis of the interaction of p53 with MDM2; -fine mapping of the MDM2 binding site on p53 using synthetic peptides. Oncogene 9, 2523-2529.

Pietenpol, J.A., Tokino, T., Thiagalingam, S., El-Deiry, W., Kinzler, K, and Vogelstein, B.(1994). Sequence-specific transcriptional activation is essential for growth suppression by p53. Proc. Natl. Acad. Sci. U.S.A. 91, 1998-2002.

Pines, J. (1995). Conformational change. Nature 376, 294-295.

Polyak, K., Lee, M-H., Erdjument-Bromage, H., Koff, A., Roberts, J., Tempst, P. and Massague, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potent mediator of extracellular antimitogenic signals. Cell 78, 59-66.

Powis, G. (1992). Signalling targets for anticancer drug development. Trends Pharmacol. Sci 12, 188-194.

Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M., Waterfield, M. D. and Downward, J. (1994). Phosphatidylinositol -OH kinase as a direct target of Ras. Nature 370, 527-532.

Sherr, C. (1994). G1 phase progression: cycling on cue. Cell. 79, 551-555.

Smythe, C., Cauldwell, B., Ferguson, M. and Cohen, P. (1988). Isolation and structural analysis of a peptide containing the novel tyrosyl-glucose linkage in glycogenin EMBO J. 7, 2681-2686.

Toyoshima, H. and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 78, 67-74.

Waldman, T., Kinzler, K. W. and Vogelstein, B. (1995). p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Research 55, 5187-5190.

Warbrick, E., Lane, D.P., Glover, D. and Cox, L. (1995). A small peptide inhibitor of DNA replication defines the site of interaction between the cyclin-dependent kinase inhibitor p21WAF1 and proliferating cell nuclear antigen. Curr. Biol. 5, 275-282.

Waga, S., Hannan, G. J., Beach, D. & Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369, 574-578.
Yu, H., Chen J.K., Feng, S., Dalgarno, D.C., Brauer, A.W. and Schreiber, S.L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. Cell 76, 933-945.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D (1993). p21 is a universal inhibitor of cyclin kinases. Nature 366, 701-704.

Zhu, L., Harlow, E. and Dynlacht, B. D. (1995). p107 uses a p21CIP1- related domain to bind cyclin/cdk2 and regulate interactions with E2F. Genes & Development 9, 1740-1752.

FIGURE 1

Peptide 1	M	S	E	P	A	G	D	V	R	Q	N	P	C	G	S	K	A	C	R	R
Peptide 2.	K	A	C	R	R	L	F	G	P	V	D	S	E	Q	L	S	R	D	C	D
Peptide 3.	S	R	D	C	D	A	L	M	A	G	C	I	Q	E	A	R	E	R	W	N
Peptide 4.	R	E	R	W	N	F	D	F	V	T	E	T	P	L	E	G	D	F	A	W
Peptide 5.	G	D	F	A	W	E	R	V	R	G	L	G	L	P	K	L	Y	L	P	T
Peptide 6.	L	Y	L	P	T	G	P	R	R	G	R	D	E	L	G	G	G	R	R	P
Peptide 7.	G	G	R	R	P	G	T	S	P	A	L	L	Q	G	T	A	E	E	D	H
Peptide 8.	A	E	E	D	H	V	D	L	S	L	S	C	T	L	V	P	R	S	G	E
Peptide 9.	P	R	S	G	E	Q	A	E	G	S	P	G	G	P	G	D	S	Q	G	R
Peptide 10.	K	R	R	Q	T	S	M	T	D	F	Y	H	S	K	R	R	L	I	F	S
Peptide 11.	T	S	M	T	D	F	Y	H	S	K	R	R	L	I	F	S	K	R	K	P

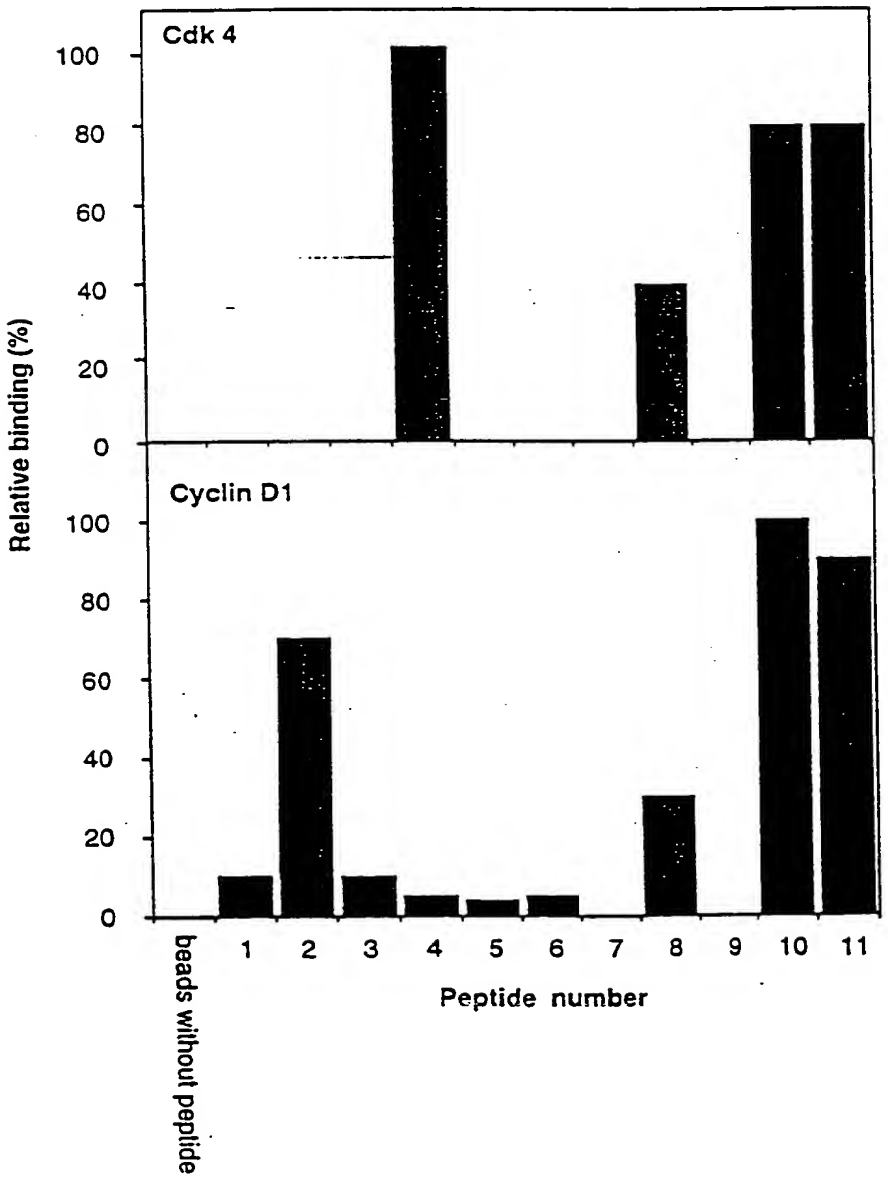
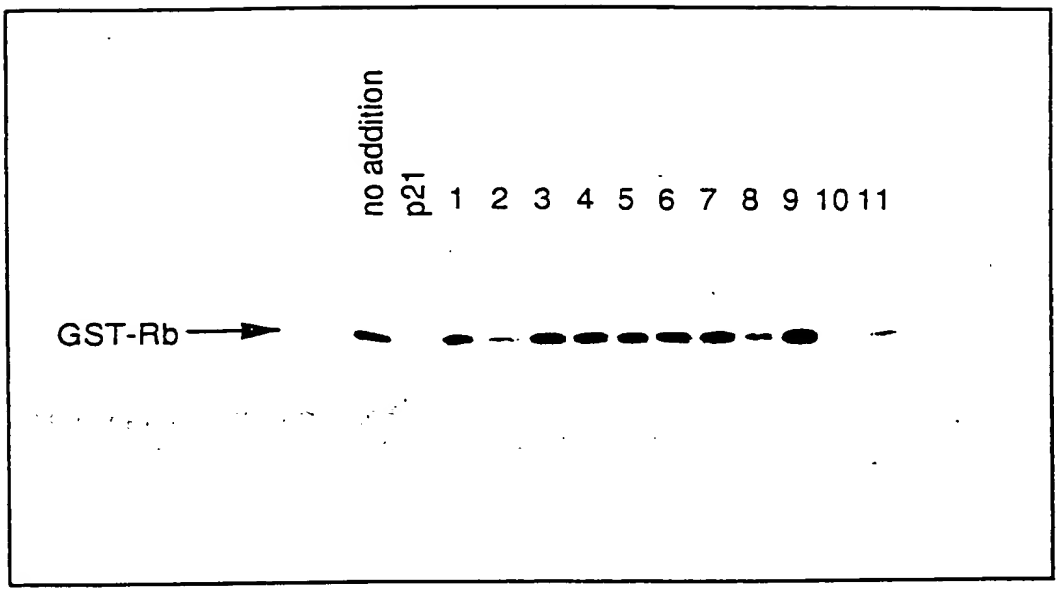


FIGURE 2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p21
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GST-Rb

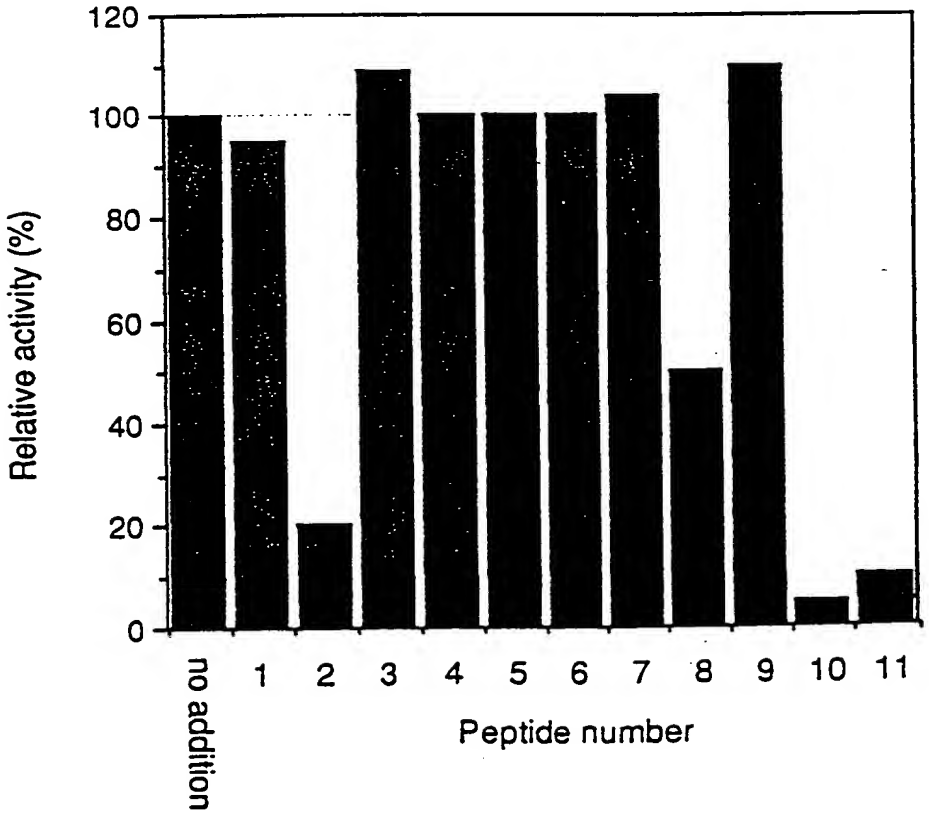
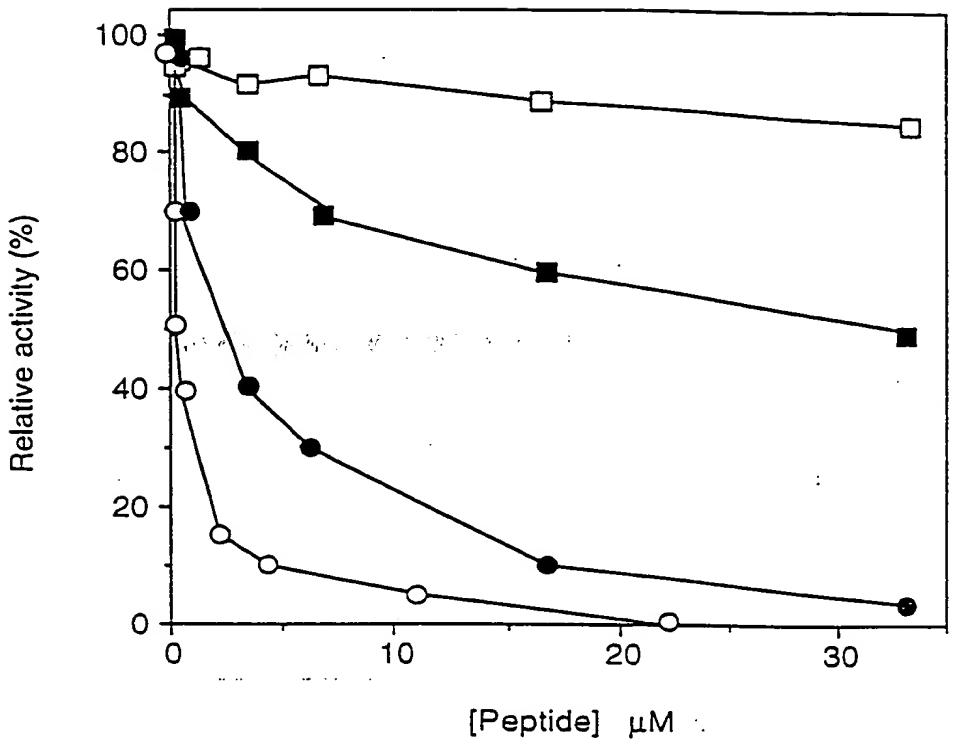
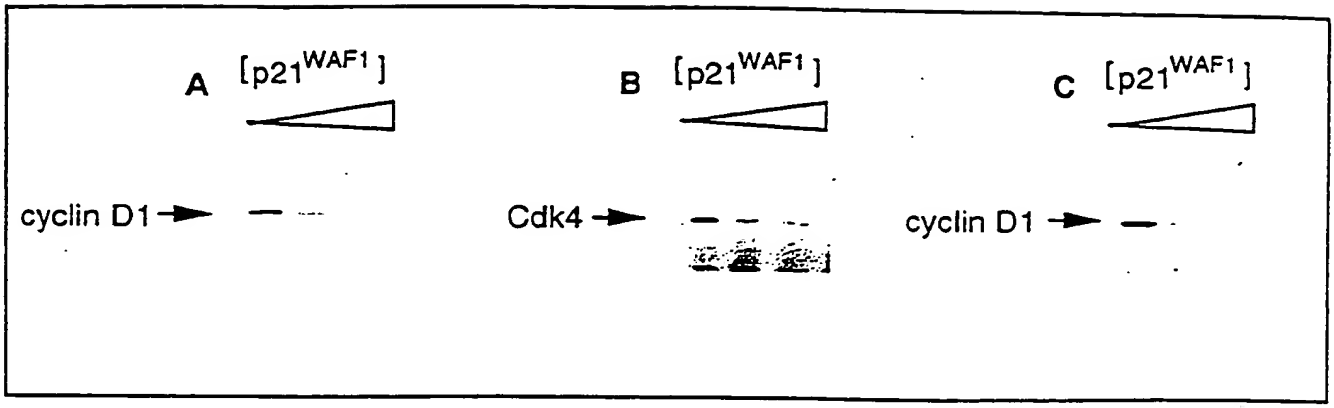

120
100
80
60
40
20
0
Relative activity (%)
no addition
1 2 3 4 5 6 7 8 9 10 11
Peptide number

FIGURE 3



Peptide number	K_i
4 (□)	---
8 (■)	> 34.0 µM
2 ( )	2.0 µM
10 (○)	0.1 µM

FIGURE 4


A
[p21WAF1]
cyclin D1
B
[p21WAF1]
Cdk4
C
[p21WAF1]
cyclin D1

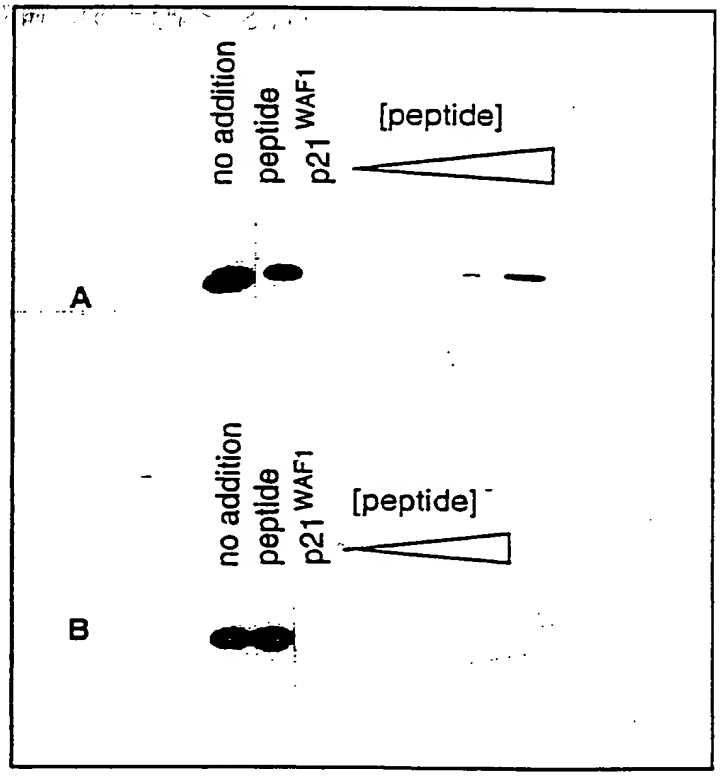
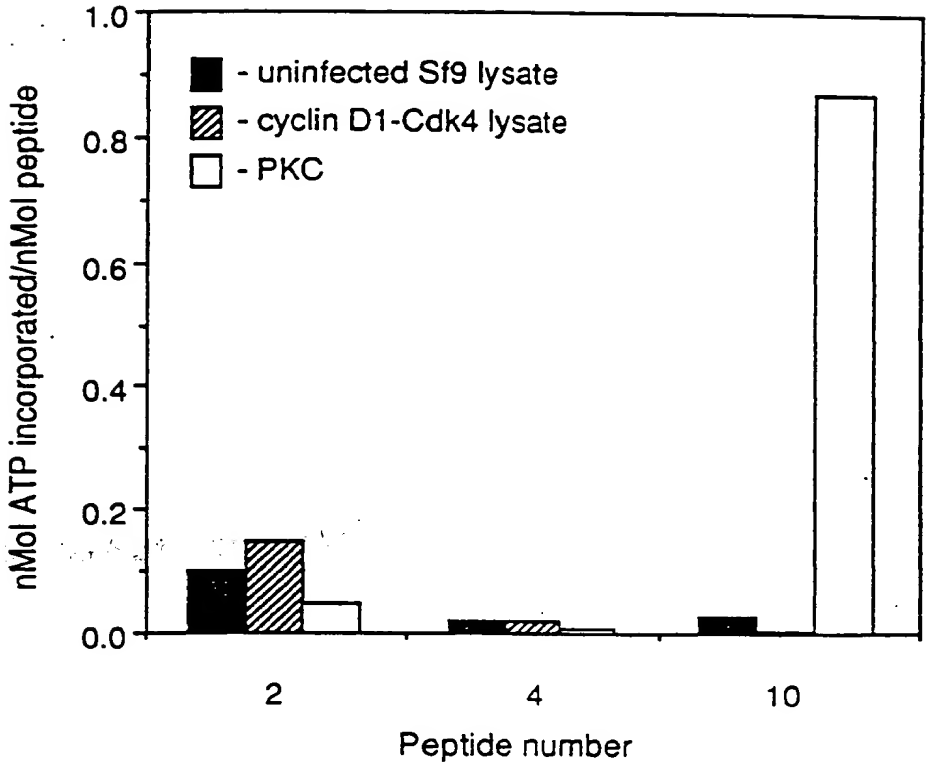

no addition
peptide p21 WAF1
[peptide]
A
no addition
peptide p21 WAF1
[peptide]
B

FIGURE 5


1.0
0.8
0.6
0.4
0.2
0.0
nMol ATP incorporated/nMol peptide
- uninfected Sf9 lysate
- cyclin D1-Cdk4 lysate
- PKC
2
4
10
Peptide number

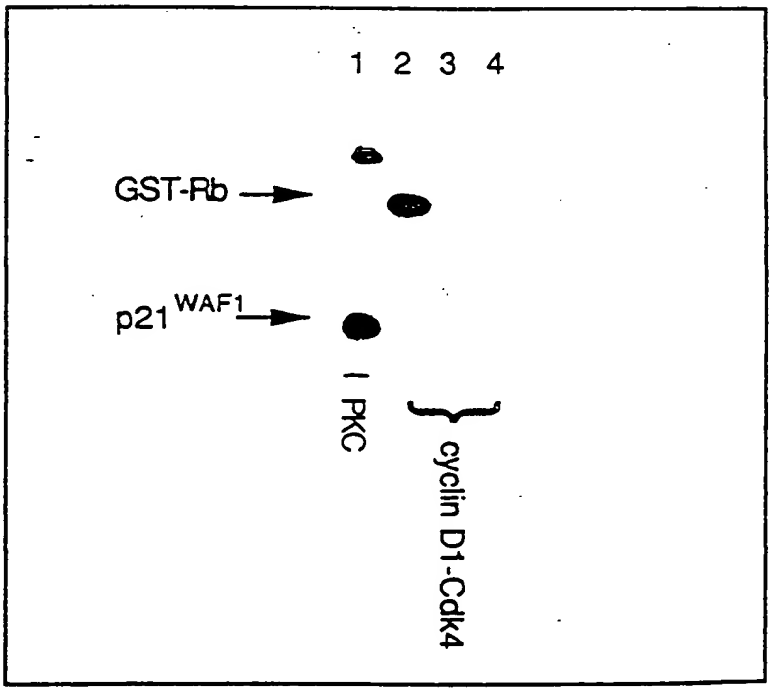

1 2 3 4
GST-Rb
p21 WAF1
PKC
cyclin D1-Cdk4

FIGURE 6

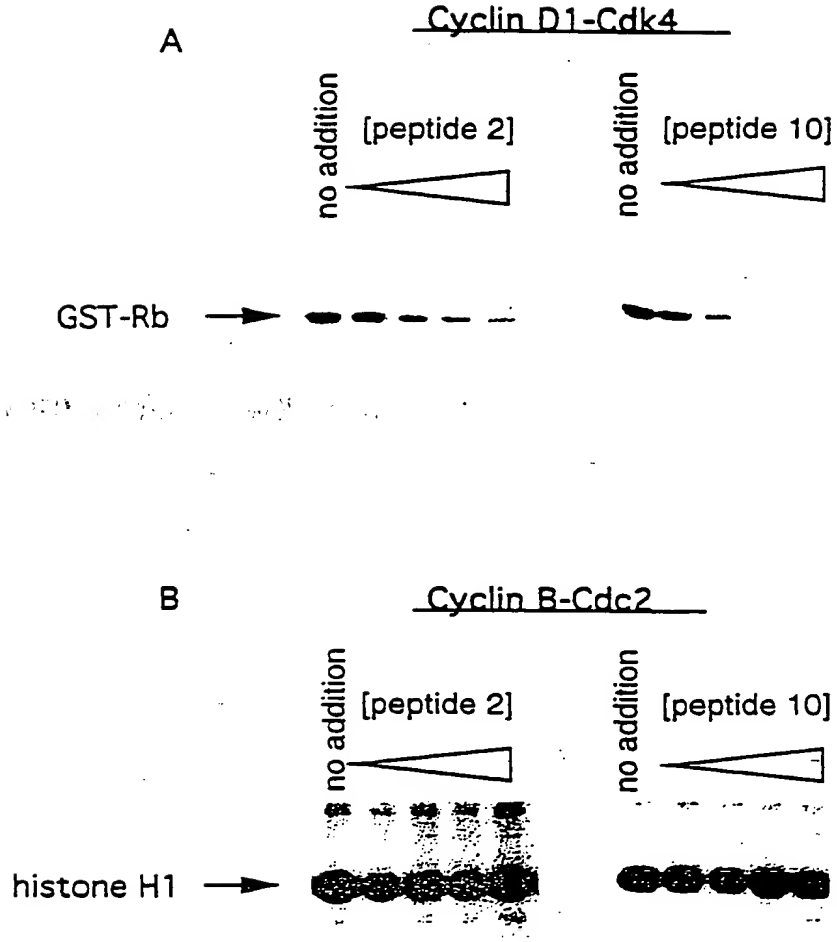

A
Cyclin D1-Cdk4
no addition
[peptide 2]
no addition
[peptide 10]
GST-Rb
B
Cyclin B-Cdc2
no addition
[peptide 2]
no addition
[peptide 10]
histone H1

FIGURE 7

a- PRSGEQAEGSPGGPGDSQGR

b- EQAEGSPGGPGDSQGRKRRQ

c- GSPGGPGDSQGRKRRQTSMT

d- GPGDSQGRKRRQTSMTDFYH

e- SQGRKRRQTSMTDFYHSKRR

peptide 10- KRRQTSMTDFYHSKRRLIFS

f- TSMTDFYHSKRRLIFSKRKP

g- DFYHSKRRLIFSKRKP

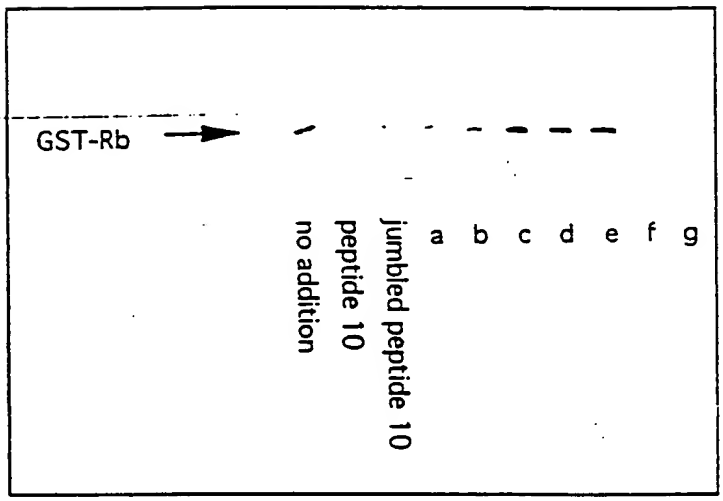


FIGURE 8

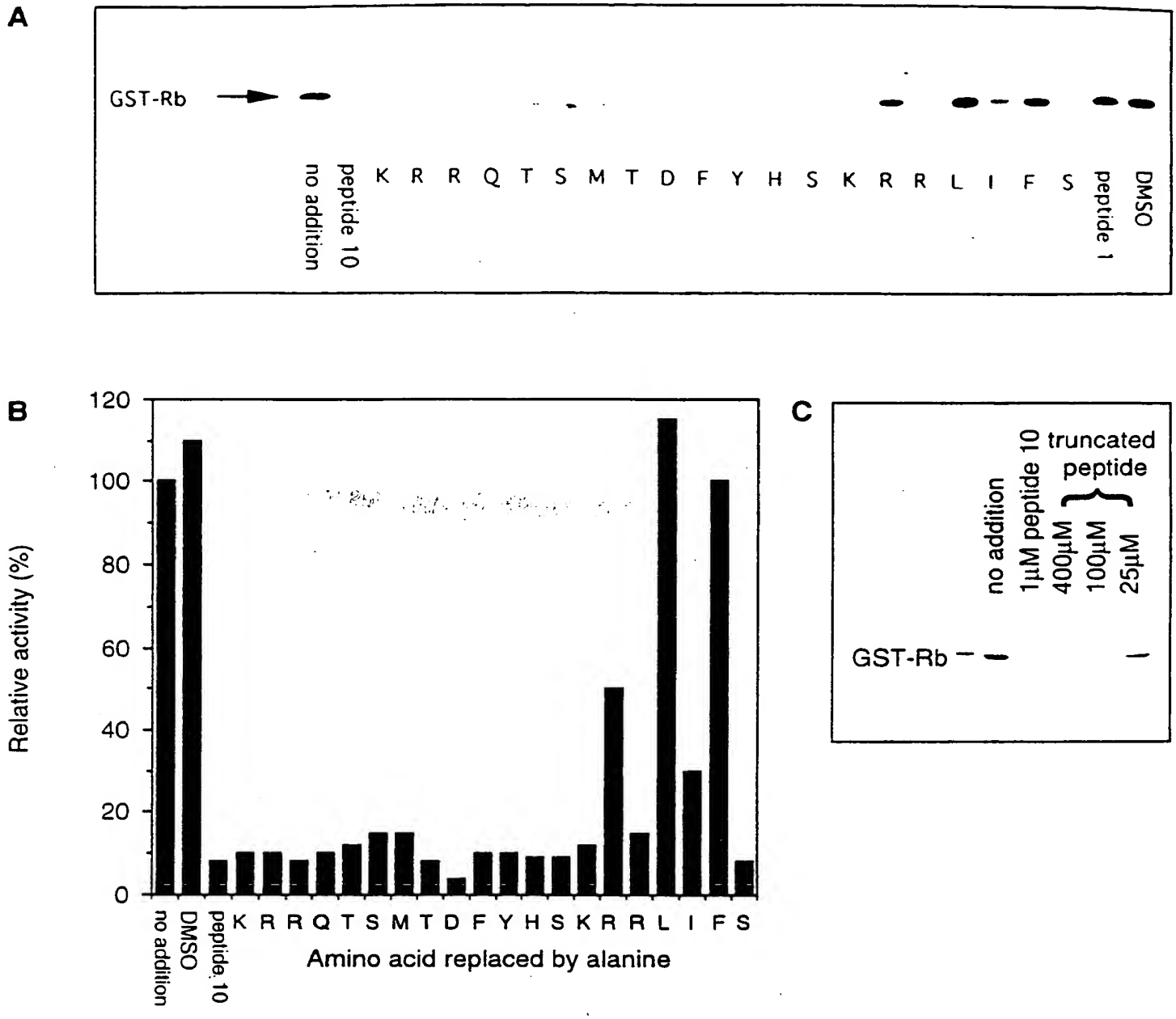
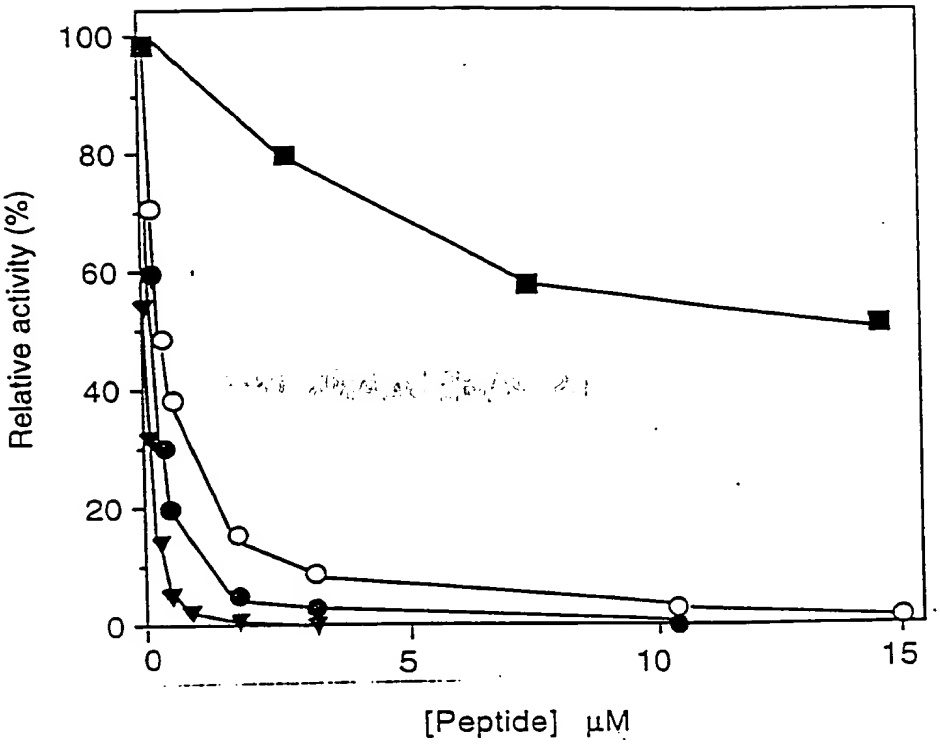

A
GST-Rb
no addition
peptide 10
K R R Q T S M T D F Y H S K R R L I F S
peptide 1
DMSO
B
Relative activity (%)
120
100
80
60
40
20
0
no addition
DMSO
peptide 10
K R R Q T S M T D F Y H S K R R L I F S
Amino acid replaced by alanine
C
no addition
1μM peptide 10
truncated peptide
400μM
100μM
25μM
GST-Rb

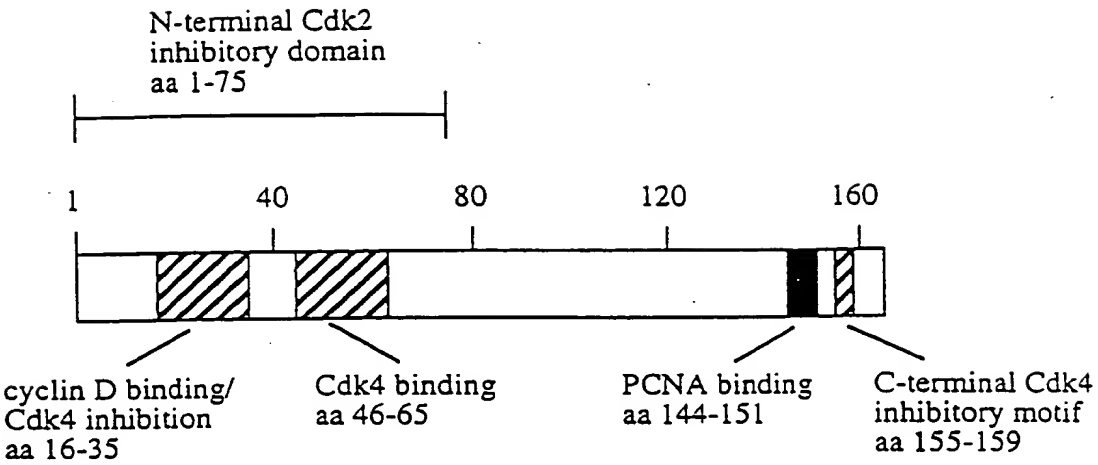
FIGURE 9



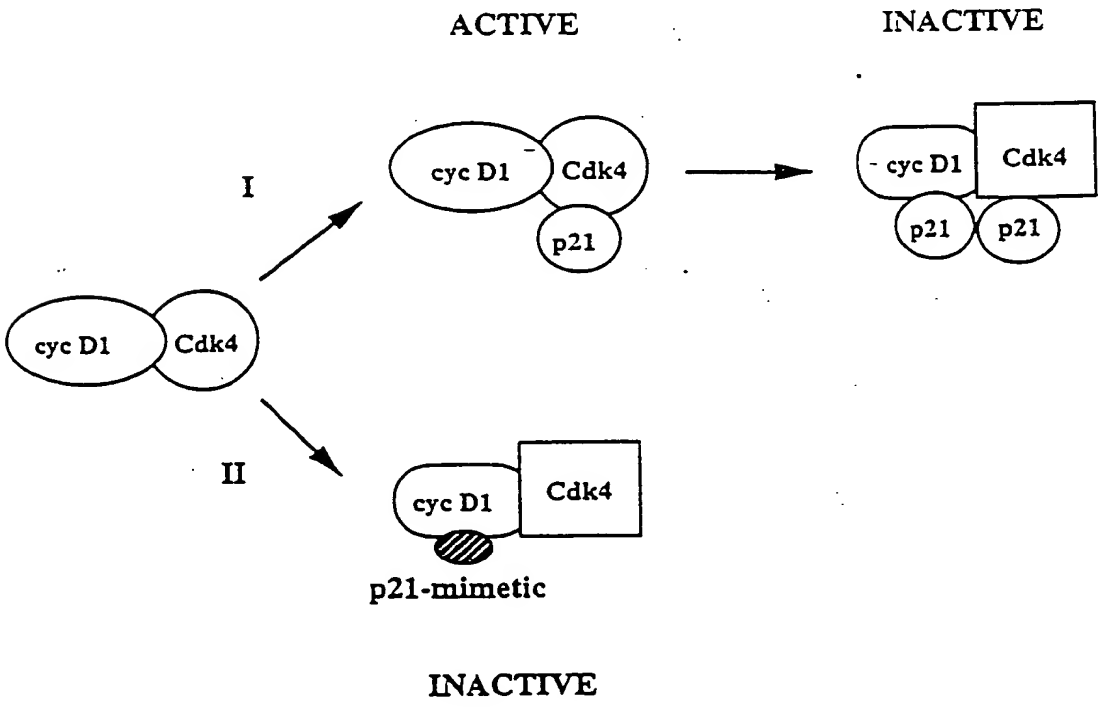
Peptide/protein	K_i
$p16^{INK4}$-peptide (■)	16.3 μM
10 (○)	0.1 μM
D-A mutant ( )	46 nM
full length $p21^{WAF1}$ (▼)	11 nM

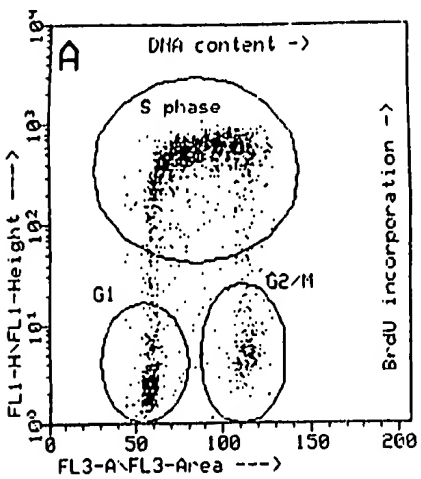
FIGURE 10

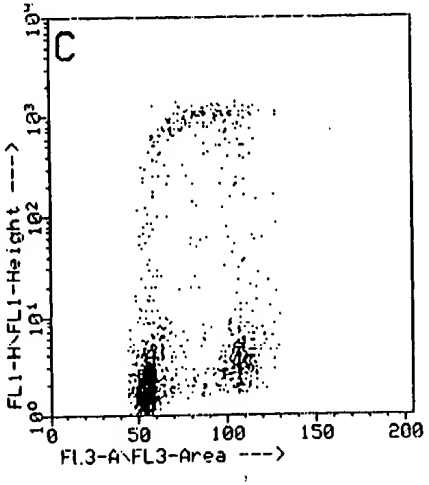
A

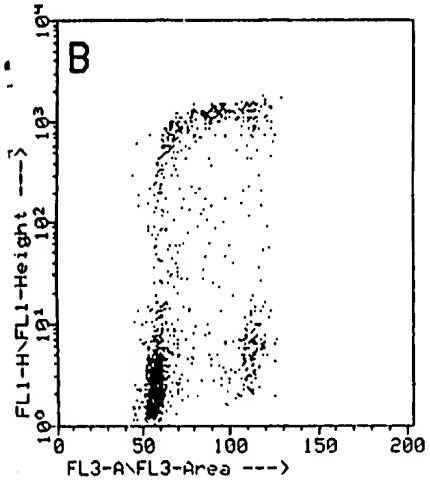


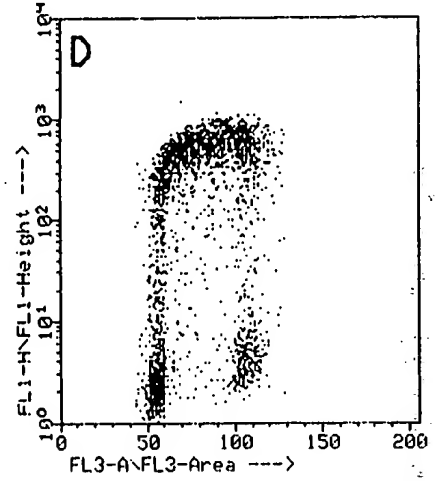
B Regulation of cyclin D1-Cdk4 activity by N-terminal p21 domains











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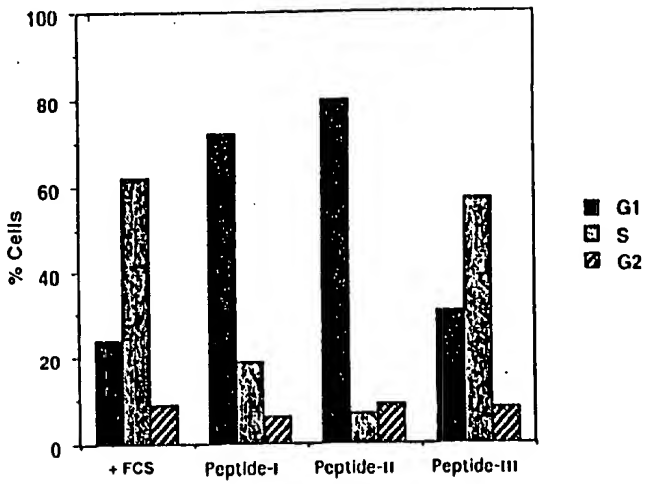

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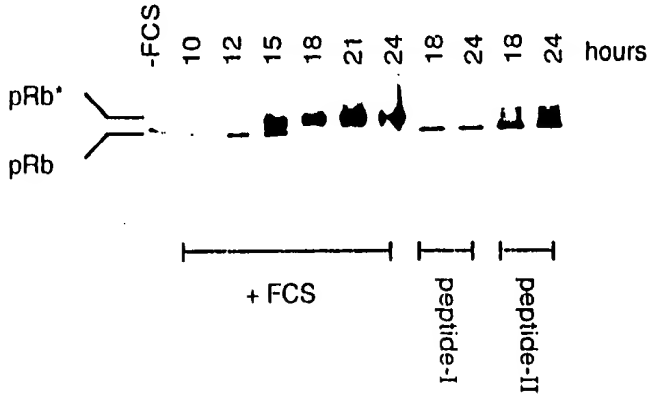

Peptide-I KRRQTSΔTDFYHSKRRLIFSRQIKIWFQNRRMKWKK

Peptide-II KRRLIFSKRQIKIWFQNRRMKWKK

Peptide-III RQTSMTDFYSHKRRRQIKIWFQNRRMKWKK

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